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TITLE: 1 a-Hydroxyvitamin D5 as a Chemotherapeutic and Possibly Chemopreventive Agent

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13. ABSTRACT (Maximum 200 Words)
We identified a novel vitamin D analog, 1α-hydroxy-24 ethyl vitamin D_5 ($1\alpha(OH)D_5$) that showed potent growth inhibitory and cell-differentiating actions in breast cancer cells. Based on our findings in vitro and in vivo, we hypothesized that $1\alpha(OH)D_5$ (D5), when administered to women with breast cancer, will induce differentiation of dedifferentiated cells and thereby prevent progression of malignancy. 1999-2000, we completed preclinical studies in rats, showing that D5 has no serious toxicity; a hypercalcemic effect was observed at high dose, which was reversible. In vitro study in tissues obtained from patients show that D5 has no effect on normal breast epithelial cells but induces apoptosis in breast cancer and showed apoptotic effect in fibroadenomas. We completed 5 steps in the synthesis of D5in preparation for phase I clinical study. 2001-2002, under GMP, we completed preclinical toxicity studies in dogs and completed synthesis of $1\alpha(OH)D_5$. In vitro studies in clinical specimens suggest that D5 has no effect on normal breast tissues; it inhibits cell proliferation in tumor cells. D5 or its active metabolite possibly interacts with estrogen receptor. 2002-2003, all the requirements for the clinical trial are completed; we submitted an application for IND to FDA. The FDA responded back with a few questions. These have been completed and the revised application is once again submitted. We will initiate studies as soon as the approval is granted. In the present report we have included the clinical portion of the brochure, since the summary of results on the efficacy and mechanism of action of $1\alpha(OH)D5$ were submitted in previous years.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
82

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Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Hypothesis proposed

We hypothesize that (1) $1\alpha(OH)D_5$ administered to women with breast cancer will induce differentiation of dedifferentiated malignant cells and thereby prevent progression of malignancy, and (2) in women with premalignant lesions, $1\alpha(OH)D_5$ will prevent dedifferentiation and thus prevent induction and/or development of breast cancer.

Technical Objectives proposed

The specific objectives of the proposed study are to:

1. Establish and evaluate biomarkers predicting $1\alpha(OH)D_5$ response in malignant breast cancer and DCIS (Ductal Carcinoma in Situ).
2. Study the molecular mechanism by which $1\alpha(OH)D_5$ induces differentiation/inhibits proliferation of breast cancer cells.
3. Perform (according to FDA requirement) preclinical toxicity and pharmacokinetic studies of $1\alpha(OH)D_5$.
4. Initiate a phase I/II trial in advanced breast cancer patients. (During this trial, we will also obtain data on the metabolism of $1\alpha(OH)D_5$ in humans.)

Goals 1-3 have already been completed. During the past year the major effort is directed towards preparing application for IND approval from the FDA. The requirements include, manufacturing of the compound $1\alpha(OH)D5$ under Good Manufacturing Practice guidelines. 2. Complete the preclinical toxicity in two sexes under Good Laboratory Practice. Identify a clinical trial monitor and identify a consultant who would assist with putting the application together. Ms. Arvilla Trag of Midwest Consulting has been serving as a consultant for the past year. Dr. Manley Paulos serves as an advisor for the clinical trial. Successful completion of the proposed study will identify a new chemotherapeutic and possibly chemopreventive agent in breast cancer.

Results

During the past year, the principal focus was on submitting an IND application to FDA and initiate clinical trials as was proposed in the last specific aim (Aim 4 of the original application). This brochure included summary of results derived on $1\alpha(OH)D5$, GMP synthesis of the compound, purity and stability of the drug, formulation, percent recovery from the capsule, stability determined under various conditions including temperature, oxygen, light and air, dissolubility of $1\alpha(OH)D5$ and preclinical toxicity. Since the entire brochure is voluminous 550 pages of the application and nearly 500 pages of preclinical toxicity studies, in this report we have included only the portions of the application not submitted as report in previous years.

The application is divided into 10 parts. **Parts 1-6** include summary f results from previous years, background information, disease relevance, Description of facilities, resumes of clinicians and clinic and IRB response. These sections are not included here. **Parts 7-10** are included in this report.

Summary of accomplishments:

- Prepared Investigator's brochure for submission to FDA for IND approval
- Prepared formulation of the 1α(OH)D5 for clinical trials
- Conducted stability, recovery and dissolubility studies with the compound.
- Completed preclinical toxicity studies with rats and dogs.
- Submitted application to FDA and received additional questions from the FDA regarding stability of the compound upon exposure to oxygen. We completed studies to successfully answer these questions.
- Once the approval is granted we will recruit patients immediately for the trial.

7. Chemistry, Manufacturing, and Control Data

7.1. Chemistry and Manufacturing Introduction

If the sponsor believes either the chemistry of the drug substance or drug product, or the manufacturing of the drug substance or drug product presents any signals of potential human risk, these signals should be discussed here. The steps proposed to monitor such risk(s) should be described, or the reasons why the signal(s) should be dismissed.

In addition, the sponsors should describe any chemistry and manufacturing differences between the drug product proposed for clinical use and the drug product used in the animal toxicology trials that formed the basis for the sponsor's conclusion that it was safe to proceed with the proposed clinical study. How these differences might affect the safety profile of the drug product should be discussed. If there are no differences in the products, that should be stated. The compound used in non-clinical studies was synthesized by Dr. Raju Penmasta, President, Synquest Inc., Chicago, IL, according to the Good Manufacturing Practice (GMP) guidelines. Drug substance for clinical studies was synthesized at Sai Drusyn Laboratories, Andhra Pradesh, India.

Synthesis of the preclinical material was as follows:

Step 1: Stigmasterol Tosylate:

Stigmasterol (50 g) was dissolved in pyridin (175 mL) and cooled in an ice bath to 0 – 5 °C. To this was added in several portions Tosyl chloride (43 g) over a period of 0.5 hours. The resulting solution was stirred at room temperature in the dark for 20 hours. Progress of the reaction was monitored by TLC (5% Hexane : EtoAc rf 0.5). The reaction mixture was poured into cold 5% $NaHCO_3$ solution, and tosylate precipitated. The solid was stirred for 15 minutes, filtered and washed with water, and air dried to yield 64 g of stigmasterol tosylate.

Step 2: Preparation of stigmasterol methyl ether:

A suspension of stigmasterol tosylate (64 g), potassium acetate (70 g) and anhydrouys methanol was refluxed for 5 hours. The reaction was monitored by TLC (Rf = 0.7, 5% Hexane : EtOAc). Mthanol was evaporated in a vacuum, ether was added and washed with water, 5% $NaHCO_3$ brine, and dried over sodium sulphate. The solvent was concentrated in a vacuum to yield 46 grams of methyl ether as a pale yellow viscous liquid.

Step 3: Preparation of sitosterol methyl ether:

Step 8: Preparation of Vitamin D5 tosylate:

Triethyl amine (2.8 mL) was added to a solution of Vitamin D5 (3.3 g) in dry methylene chloride (100 mL). The mixture was cooled to 0 °C. After 15 minutes of stirring tosylchloride (3.0 g) was added and the reaction was brought to room temperature and stirred for 3 hours. The progress of the reaction was monitored by TLC. Saturated sodium bicarbonate was added and extracted with dichloromethane, washed with brine and water, and dried to yield the tosylate as a syrupy compound (4.12 g).

Step 9: Preparation of cyclovitamin D5:

The above tosylate was added to methanol (180 mL) and saturated sodium bicarbonate (4.1.1 g) and was refluxed for 5 hours. Progress of the reaction was monitored by TLC. The solvent was removed under vacuum and poured into cold water. The product was extracted into dichloromethane. The organic layer was washed with brine solution and dried over sodium sulfate and evaporated to yield methyl ether (3.2 g).

Step 10: Preparation of 1α-hydroxycyclovitamin D5:

A mixture of selenium oxide (0.46 g), TBHP (1.48 g), and dichloromethane (100 mL) was stirred for 3 hours under nitrogen at room temperature. The mixture was cooled to 0 °C and a catalytic amount of pyridine was added. The methyl ether (3.2 g) dissolved in dichloromethane (30 mL) was added by drops over 15 minutes and then stirred for 1 hour. The progress of the reaction was monitored every 10 minutes. The crude mixture was purified by column chromatography to yield 1.1 g of allyl alcohol derivative of vitamin D5.

Step 11: Preparation of cis and trans mixture of 1α-hydroxyvitamin D5:

The above compound (1.05 g) was stirred in a mixture of DMSO and acetic acid at 56 – 60 °C under nitrogen in a water bath. After 1 hour TLC showed the completion of the reaction. The mixture was poured into water and extracted with ethyl acetate, and concentrated to yield 1.0 g of product.

Step 12: Preparation of 1α-hydroxyvitamin D5:

A solution of the crude product (1.0 g), maleic anhydride (230 mg) , and ethyl acetate (160 mL) was stirred at room temperature for 24 hours under nitrogen. The solvent was stripped off under vacuum and chromatographed over silica gel and eluted with ethyl acetate and hexanes to yield 500 mg of product. The product was further purified by reverse phase HPLC, followed by crystallization from hexane to yield 350 mg of 1α-hydroxyvitamin D5 with a purity > 98%.

Step 7: Preparation of Vitamin D5:

The crude vitamin D5 acetate (6.3 g) was dissolved in dry THF (250 mL) and cooled to 0 °C under stirring. Lithium aluminum hydride (5.27 g) was added slowly in several portions over a 30 minute period and stirred at room temperature for 1.5 hours. Progress of the reaction was monitored by TLC. The reaction was quenched by slow addition of water and diluted with ethyl acetate. The mixture was filtered through a celite filter and the residue was washed with ethyl acetate. The combined solvents were evaporated to yield crude Vitamin D5. Column purification of the crude D5 yielded 3.3 g of pure compound.

Step 8: Preparation of Vitamin D5 tosylate:

Triethyl amine (2.8 mL) was added to a solution of Vitamin D5 (3.3 g) in dry methylene chloride (100 mL). The mixture was cooled to 0 °C. After 15 minutes of stirring tosylchloride (3.0 g) was added and the reaction was brought to room temperature and stirred for 3 hours. The progress of the reaction was monitored by TLC. Saturated sodium bicarbonate was added and extracted with dichloromethane, washed with brine and water, and dried to yield the tosylate as a syrupy compound (4.12 g).

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The above compound (1.05 g) was stirred in a mixture of DMSO and acetic acid at 56 – 60 °C under nitrogen in a water bath. After 1 hour TLC showed

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Step 12: Preparation of 1α-hydroxyvitamin D5:

A solution of the crude product (1.0 g), maleic anhydride (230 mg) , and ethyl acetate (160 mL) was stirred at room temperature for 24 hours under nitrogen. The solvent was stripped off under vacuum and chromatographed over silica gel and eluted with ethyl acetate and hexanes to yield 500 mg of product. The product was further purified by reverse phase HPLC, followed by crystallization from hexane to yield 350 mg of 1α-hydroxyvitamin D5 with a purity > 98%.

7.2. Drug Substance

7.2.1. A description of the drug substance, including its physical, chemical, or biological characteristics.

1α-Hydroxyvitamin D5 is a white powder with a molecular formula of $C_{29}H_{48}O_2$ and a molecular weight of 428.7. It has a melting point of 142 – 143 °C. The drug substance is soluble in common organic solvents, such as methanol, ethanol, acetonitrile, acetone, chloroform, and is insoluble in water.

7.2.2. The name and address of its manufacturer.

Sai Drusyn Laboratories, Ltd.
H.No. 11-15-12/4
Siris Complex, Doctors Colony Road
L.B. Nagar
Hyderabad 500035
Andhra Pradesh
India
Tel: 91-40-4035646
Fax: 91-40-40335889

7.2.3. The general method of preparation of the drug substance.

The drug substance, Vitamin D5, is prepared from stigmasterol in seven steps. The Process Flow Diagram and a schematic of the chemical reaction for the drug substance are included on the following pages. The following components are used in the manufacture of the drug substance:

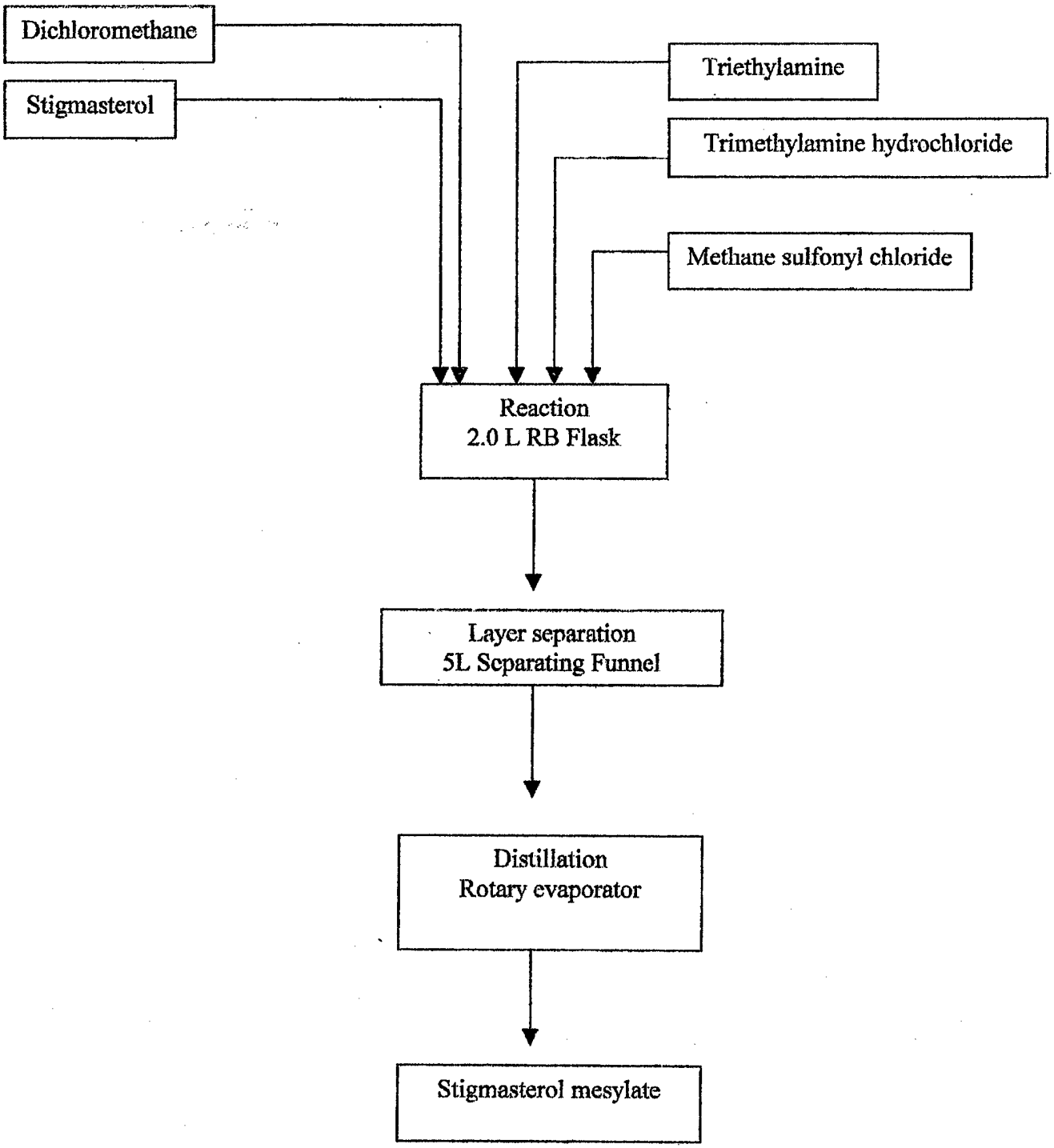
Methylene chloride
Acetonitrile
Triethylamine
Tosyl chloride
Sodium bicarbonate
Sodium chloride
Methanol
Sodium sulfate
Selenium oxide
Pyridine
Ethyl acetate
Hexanes
Dimethylsulfoxide (DMSO)
Acetic acid
Silica gel
Maleic anhydride

001--395

7.2.4. Drug Substance Process Flow Diagram

1α-Hydroxy Vitamin D_5

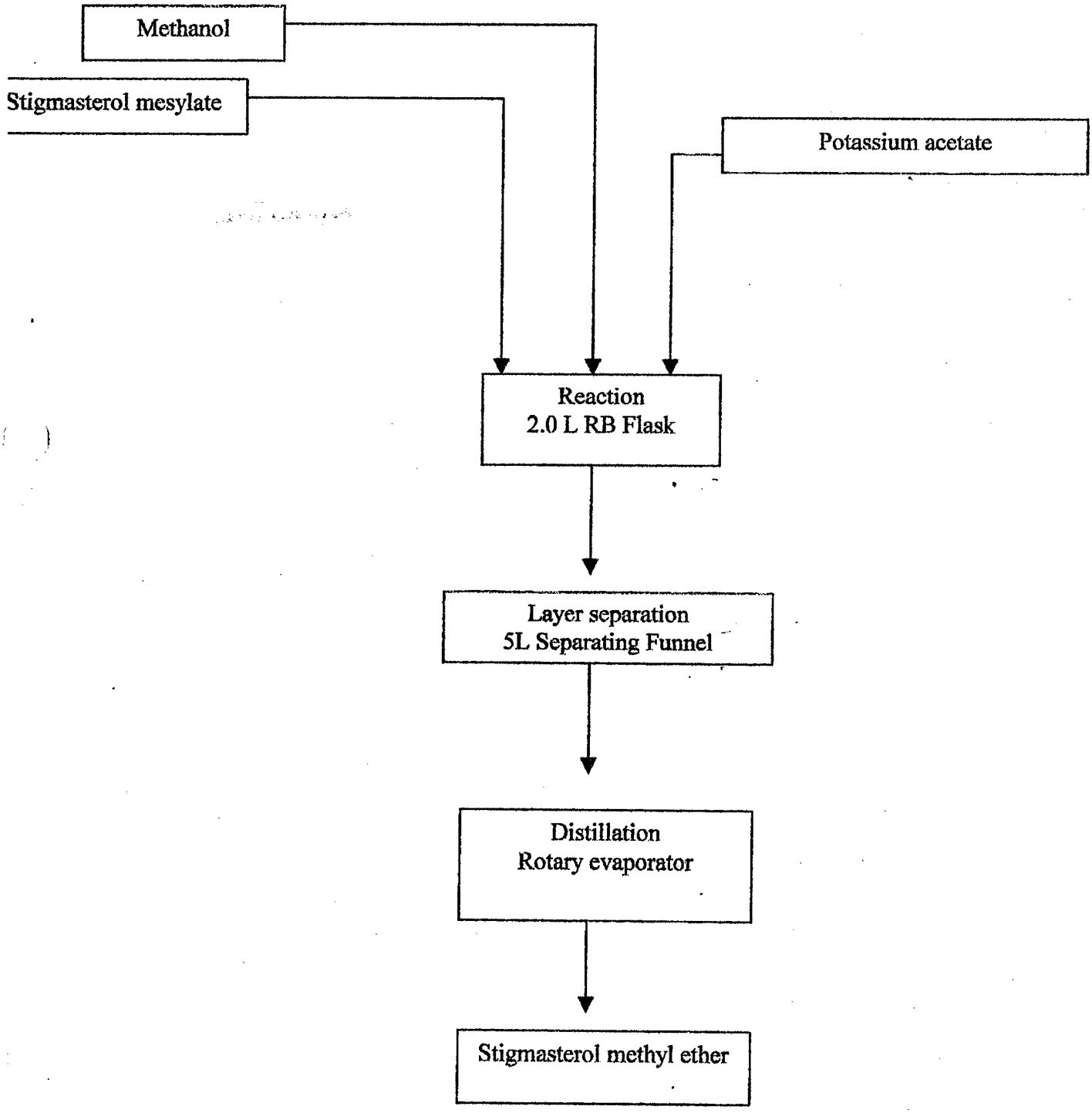
Stage-1: Stigmasterol to stigmasterol mesylate **Batch size: 100 g**



001--397

1α-Hydroxy Vitamin D_5

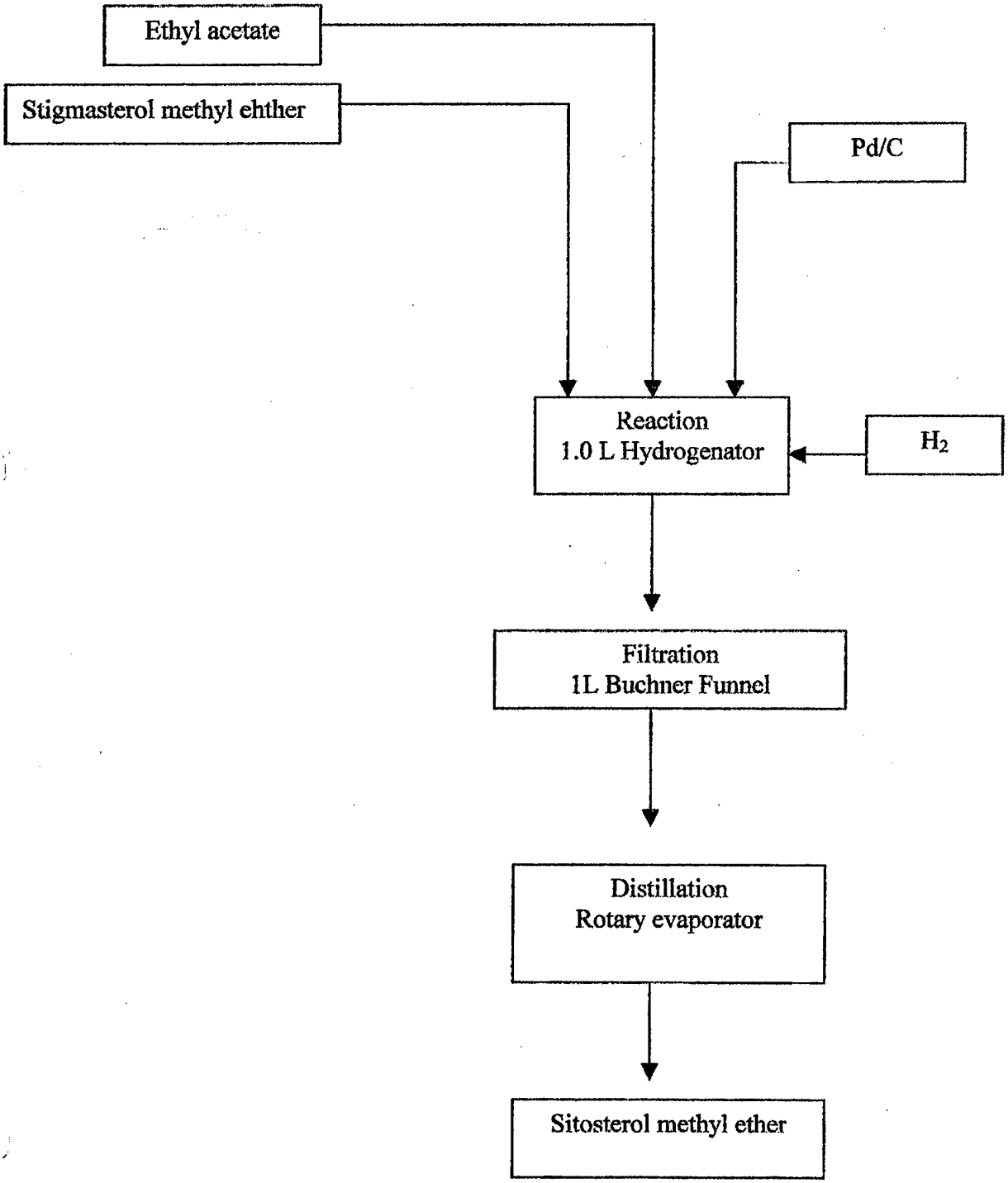
Stage-2: Stigmasterol mesylate to Stigmasterol methyl ether Batch size: 100 g



001--398

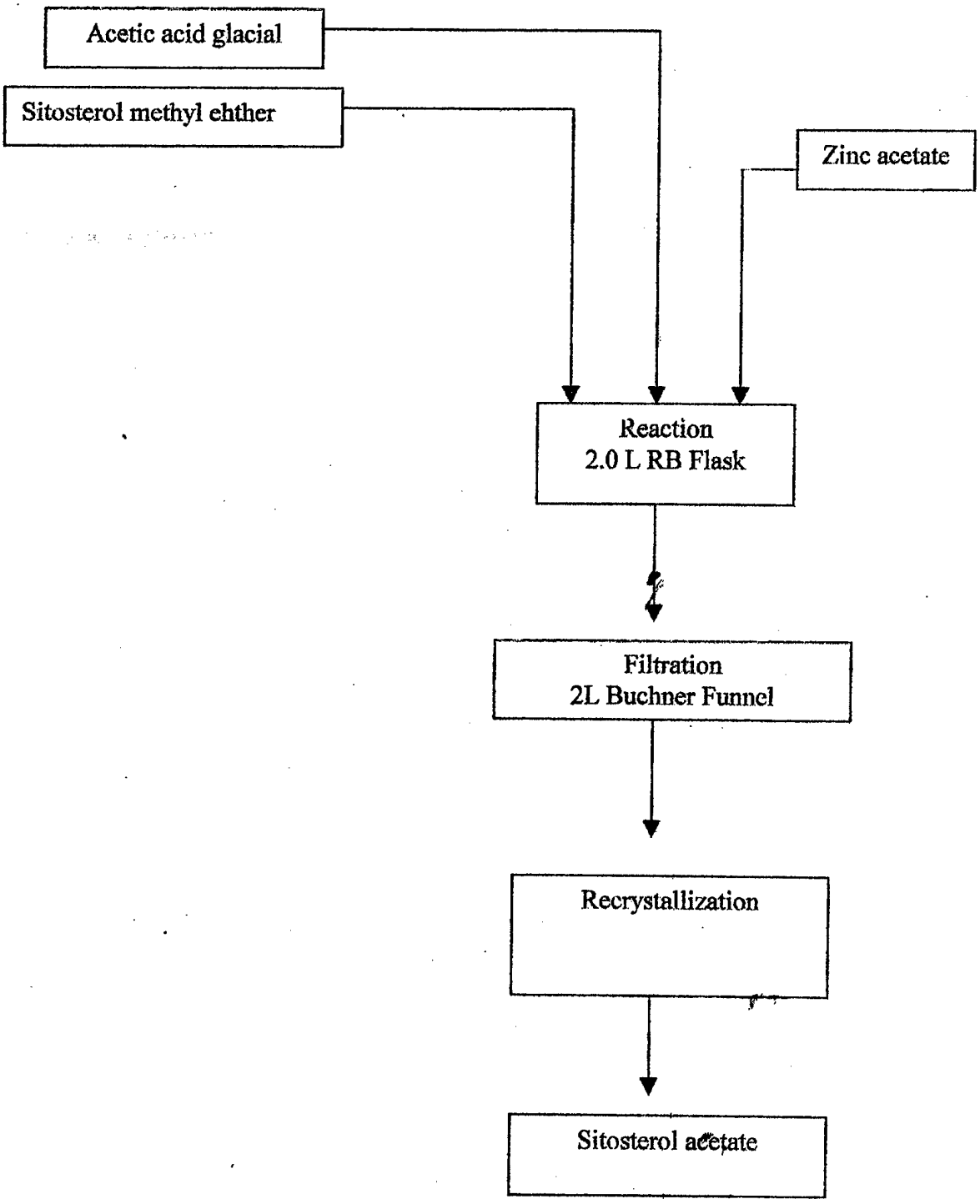
1α-Hydroxy Vitamin D_5

Stage-3: Sigmasterol methyl ether to Sitosterol methyl ether **Batch size: 100 g**



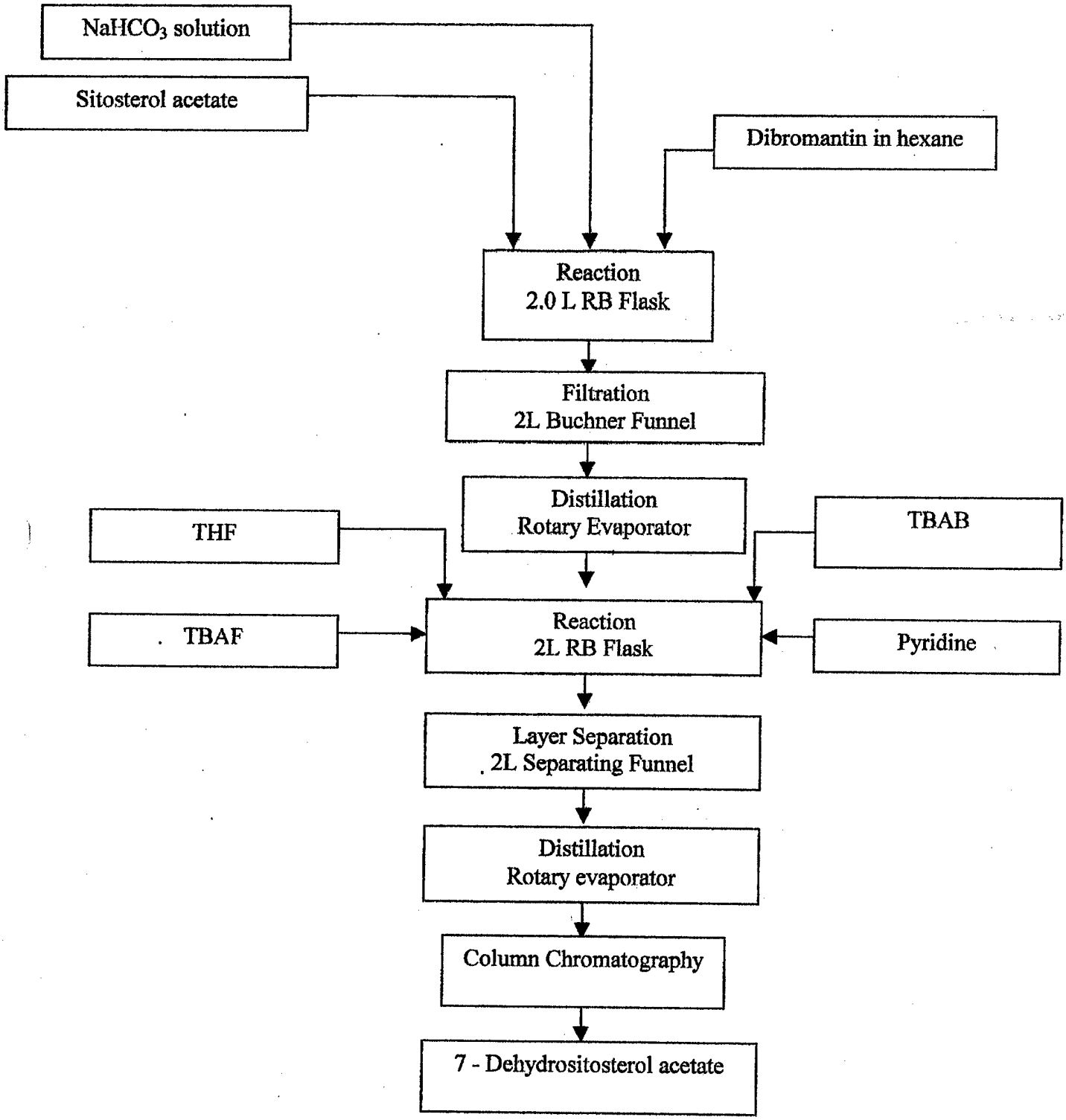
1α-Hydroxy Vitamin D_5

Stage-4: Sitosterol methyl ether Sitosterol acetate **Batch size: 100 g**



1α-Hydroxy Vitamin D_5

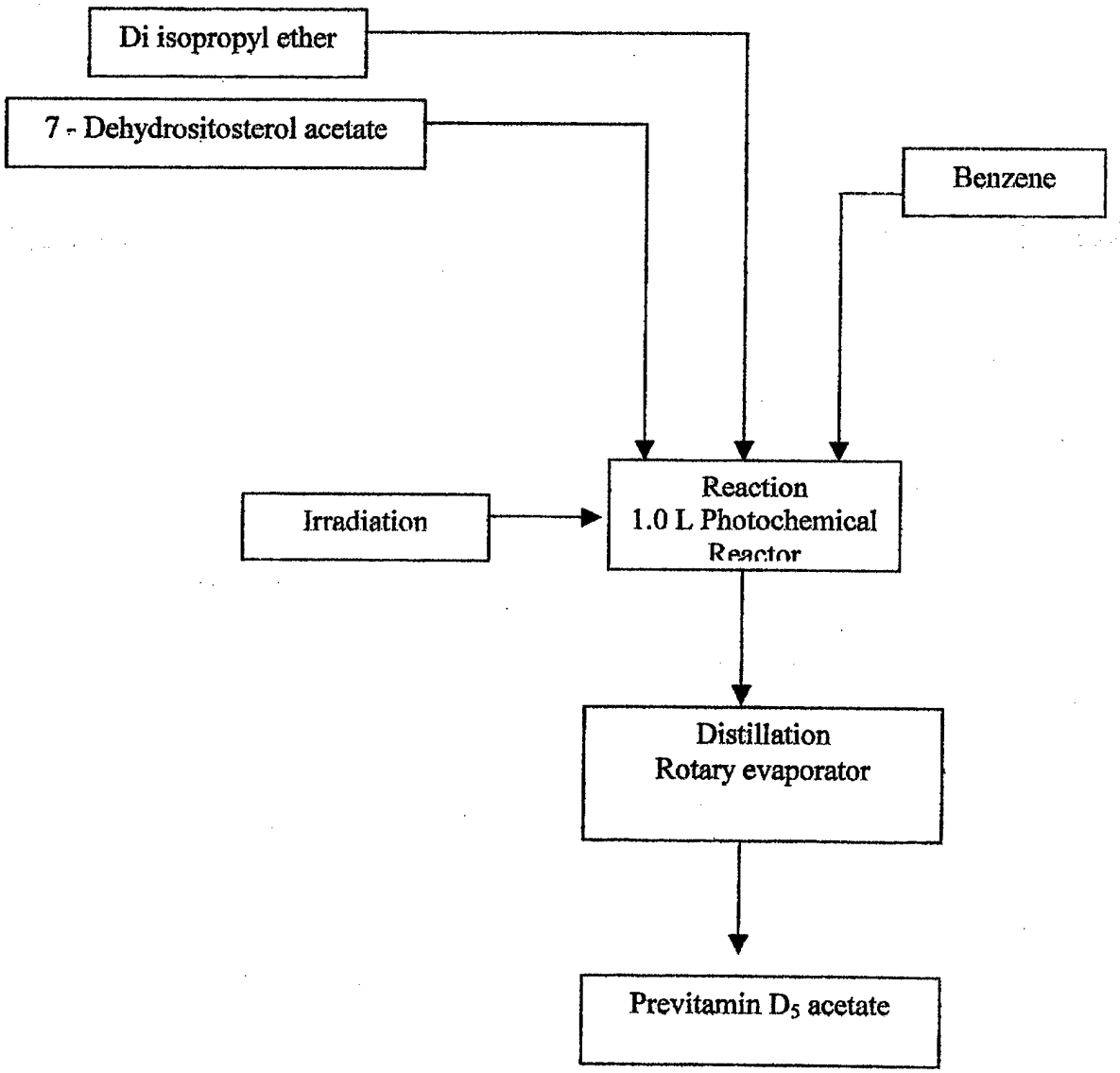
Stage-5: Sitosterol acetate to 7-Dehydrositosterol acetate **Batch size: 100 g**



001--401

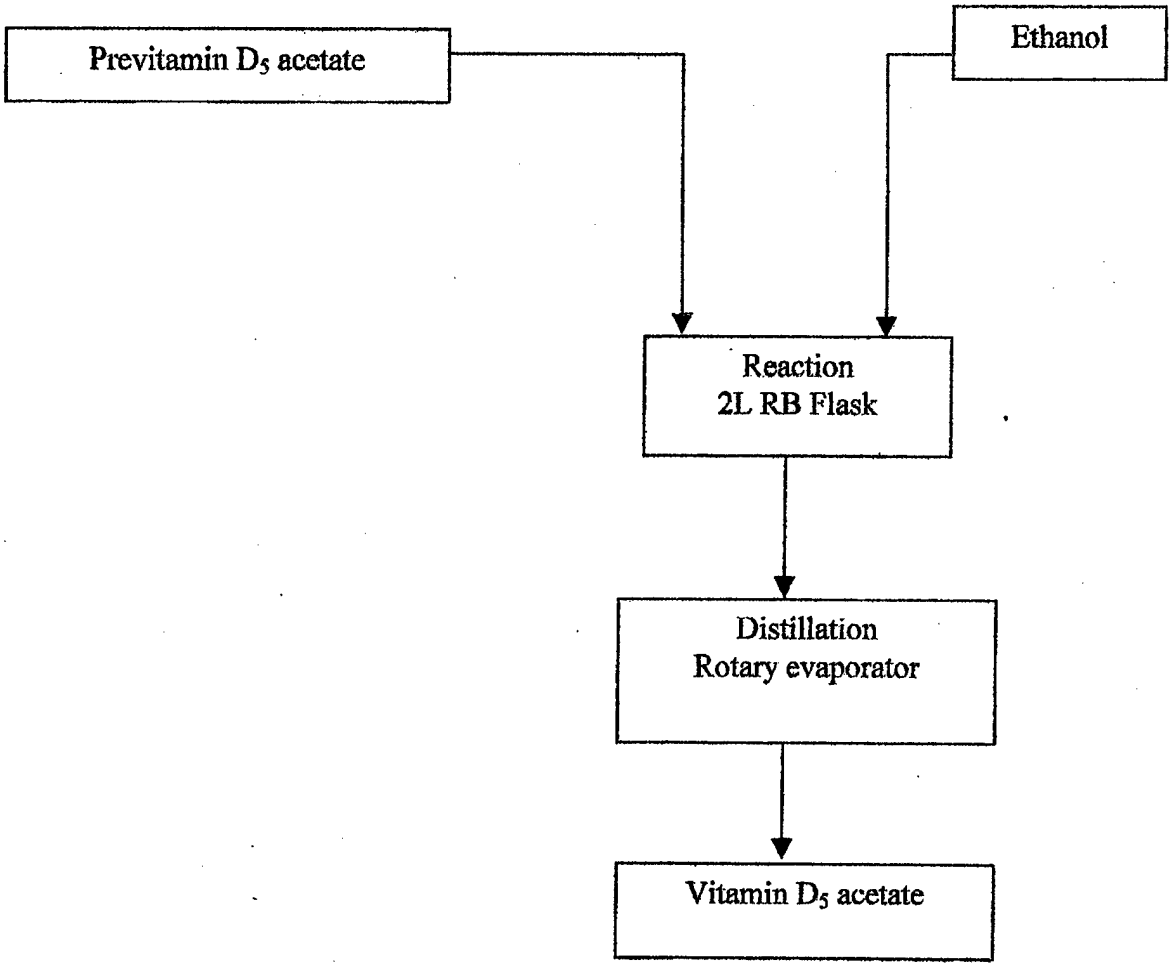
1α-Hydroxy Vitamin D_5

Stage-6: 7-Dehydrositosterol acetate to Previtamin D_5 acetate Batch size: 10 g



1α-Hydroxy Vitamin D_5

Stage-7: Previtamin D_5 acetate to Vitamin D_5 acetate **Batch size: 10 g**



7.3.4. The acceptable limits and analytical methods used to assure the identity, strength, quality and purity of the drug product.

Samples of drug product will be analyzed after formulation of each batch of capsules, using a Waters HPLC System.

Mobile phase: Acetonitrile:Methanol:Water (60:30:10)
Flow Rate: 1 mL/ minute
Wavelength: 254 nm
Column: Rainin Microsorb C18, 5 micron
Vitamin D5 resolution: 36 minutes

7.3.5. Information to support the stability of the drug product during the toxicological studies and the proposed clinical study(ies).

DETERMINATION OF 1α-HYDROXYVITAMIN-D5 MIXED IN CORN STARCH

Extraction Procedure:

500 mg of drug product sample containing 25ng of D5 (50 mG/kg) was extracted with 5 ml of HPLC grade Acetonitrile in an 8 ml vial by vortex mixing for 30 seconds, after which further extraction was done by placing the sample on a Roto-Mix rocking platform for one hour.

2 ml of the Acetonitrile extract were filtered using a 0.2 micron syringe filter. 100 microliters were used for HPLC analysis.

HPLC Conditions:

Samples were analyzed on a Waters Millenium HPLC system

Mobile phase: Acetonitrile:Methanol:Water (60:30:10)
Flow Rate: 1ml/min
Wavelength: 254nm
Column: Rainin Microsorb C18, 5 micron
Vitamin D5 resolution: 36 minutes

Limit of detection:

Based on the current resolution obtained, 0.5nG D5 in 100 μL can be used as a limit of detection for D5 in the drug product.

Vitamin D5 in Corn Starch Recovery and Stability Rates at Room Temperature

Extract Day	% Recovery from corn starch*	Degradation
Day 0	87	ND**
Day 1	87	ND
Day 2	85	ND
Day 3	82	ND
Day 4	83	ND
Day 5	80	ND
Day 7	72	ND

*= The amount of D5 recovered from the corn starch in relation to control sample of D5. The procedures could be improved to get a better recovery. No degradation was observed of D5 during this time.

**= Not Detected

001--422

Figure 1: HPLC Chromatogram of Cornstarch Blank

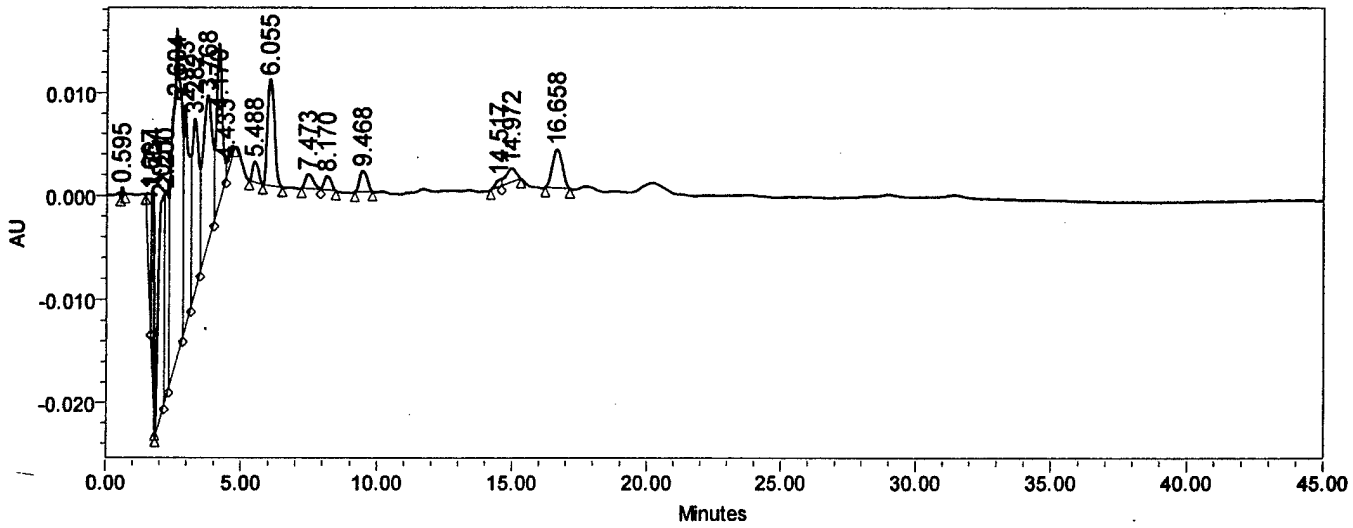
Sample Report

Reported by User: System

Project Name: Vit_D1

SAMPLE INFORMATION

Sample Name:	Diet blank	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	2/12/03 3:27:35 PM
Vial:	13	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	2/12/03 4:12:49 PM
Injection Volume:	100.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	486
Sample Set Name:	D5 diet day 0	Proc. Chnl. Descr.:	



	Peak Name	RT	Area	% Area	Height
1		0.595	3774	0.12	842
2		1.667	79652	2.53	13167
3		1.734	123272	3.91	18514
4		2.051	312676	9.92	20883
5		2.200	195969	6.22	20204
6		2.604	768648	24.40	32115
7		2.923	319013	10.12	21743
8		3.282	298621	9.48	17027
9		3.768	347037	11.01	14565
10		4.170	229101	7.27	15737
11		4.433	18891	0.60	1511
12		5.488	30122	0.96	2002
13		6.055	184079	5.84	10370

Sample Report

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
14		7.473	29658	0.94	1416
15		8.170	23866	0.76	1349
16		9.468	39964	1.27	2055
17		14.517	10933	0.35	692
18		14.972	37218	1.18	1373
19		16.658	98300	3.12	3762
20	MDA	18.000			

Figure 2: HPLC Chromatogram of D5 Standard, Day 0

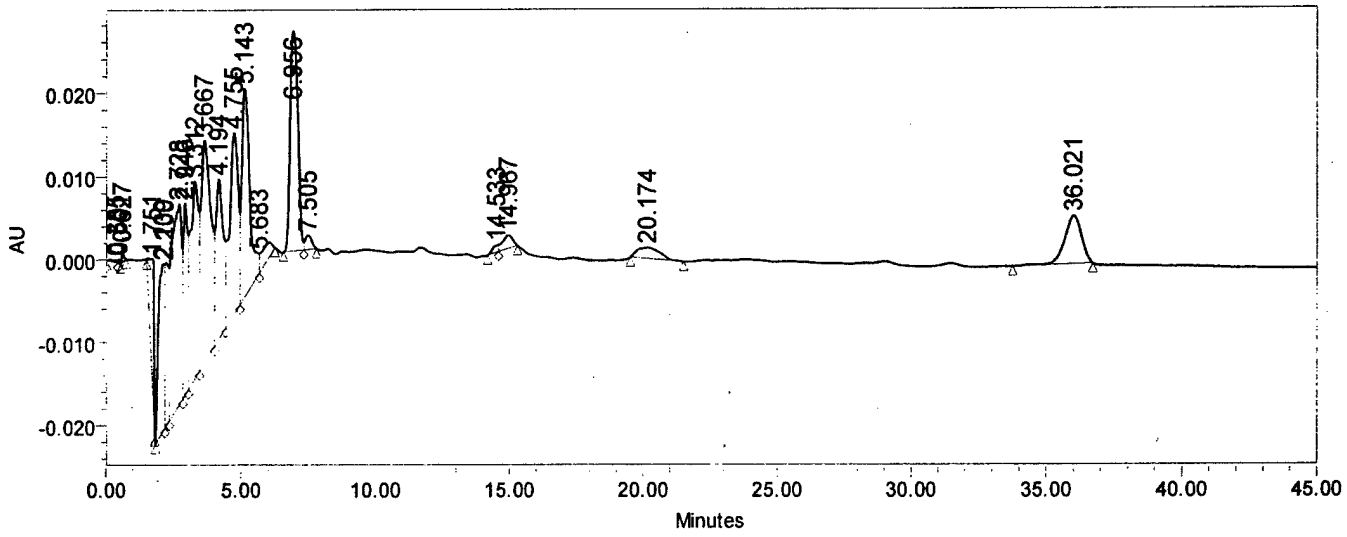
Sample Report

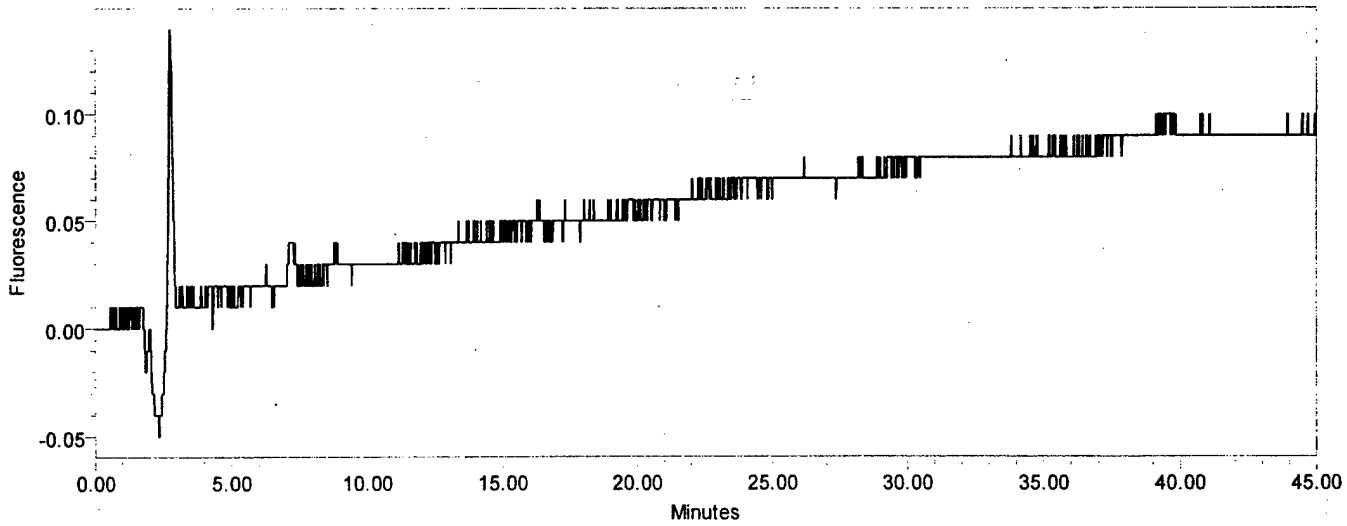
Reported by User: System

Project Name: Vit_D1

SAMPLE INFORMATION

Sample Name:	D5 std	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	2/13/03 10:50:41 AM
Vial:	12	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	2/21/03 1:00:06 PM
Injection Volume:	100.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	474 Ch1, 486
Sample Set Name:	D5 day7	Proc. Chnl. Descr.:	





	Peak Name	RT	Area	% Area	Height
1		0.385	5321	0.10	348

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
2		0.501	2246	0.04	489
3		0.627	8595	0.17	1883
4		1.751	168922	3.26	16641
5		2.139	321667	6.21	20220
6		2.200	192888	3.72	19810
7		2.728	638507	12.32	24279
8		2.946	268769	5.19	23157
9		3.312	515961	9.96	23900
10		3.667	687136	13.26	26898
11		4.194	377511	7.29	19414
12		4.755	482298	9.31	21973
13		5.143	514082	9.92	25195
14		5.683	58130	1.12	2415
15		6.956	524665	10.13	26296
16		7.505	30655	0.59	1734
17		14.533	13187	0.25	860
18		14.967	42976	0.83	1613
19	MDA	18.000			
20	MDA	18.000			
21		20.174	75701	1.46	1306
22		36.021	252242	4.87	5786

Figure 3: HPLC Chromatogram of D5 in Cornstarch, Day 0

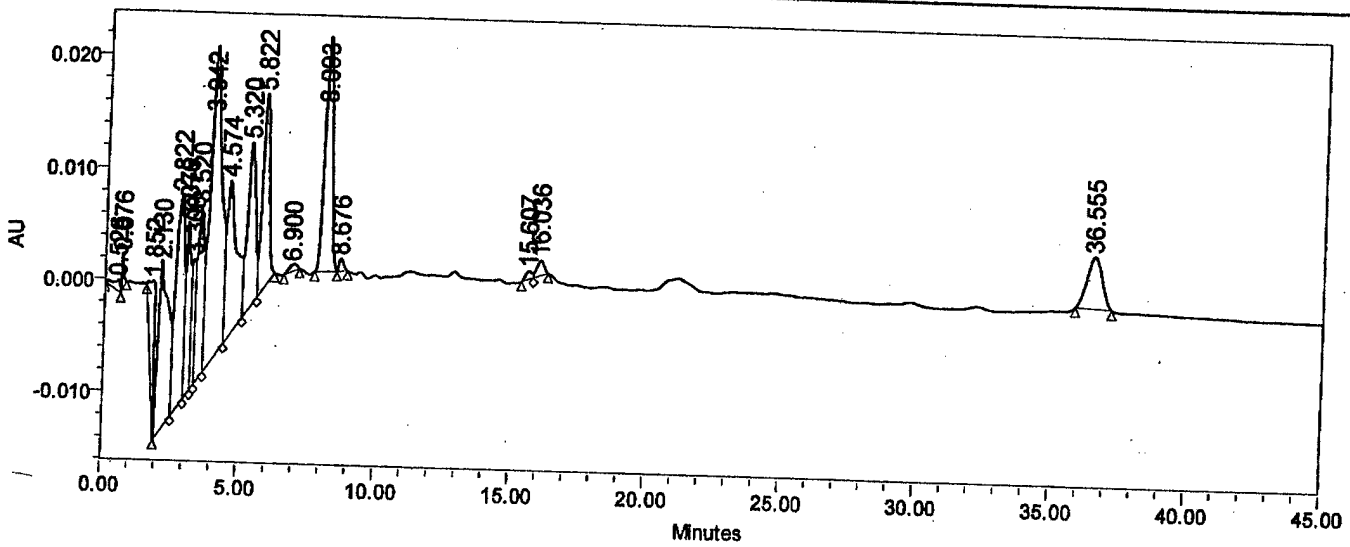
Reported by User: System

Sample Report

Project Name: Vit_D1

SAMPLE INFORMATION

Sample Name:	D5 std 2.5ugml	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	2/12/03 2:40:12 PM
Vial:	12	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	2/12/03 3:25:27 PM
Injection Volume:	100.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	486
Sample Set Name:	D5 diet day 0	Proc. Chnl. Descr.:	



	Peak Name	RT	Area	% Area	Height
1		0.526	18103	0.47	1026
2		0.676	15320	0.40	3252
3		1.852	129707	3.37	11035
4		2.130	371574	9.66	15192
5		2.822	392412	10.20	18856
6		3.076	181713	4.72	15609
7		3.308	101437	2.64	11390
8		3.520	245470	6.38	15400
9		3.942	792144	20.59	28204
10		4.574	364086	9.46	14052
11		5.320	291033	7.56	14828
12		5.822	302245	7.85	17365
13		6.900	12554	0.33	597

Report Method: Result Set Report

Printed 1:12:06 PM 2/21/03

001--430

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
14		8.093	396879	10.31	20875
15		8.676	13757	0.36	1138
16		15.607	12430	0.32	741
17		16.036	25433	0.66	1351
18	MDA	18.000			
19		36.555	181793	4.72	4688

001--431

Figure 4: HPLC Chromatogram of D5 in Cornstarch, Day 7

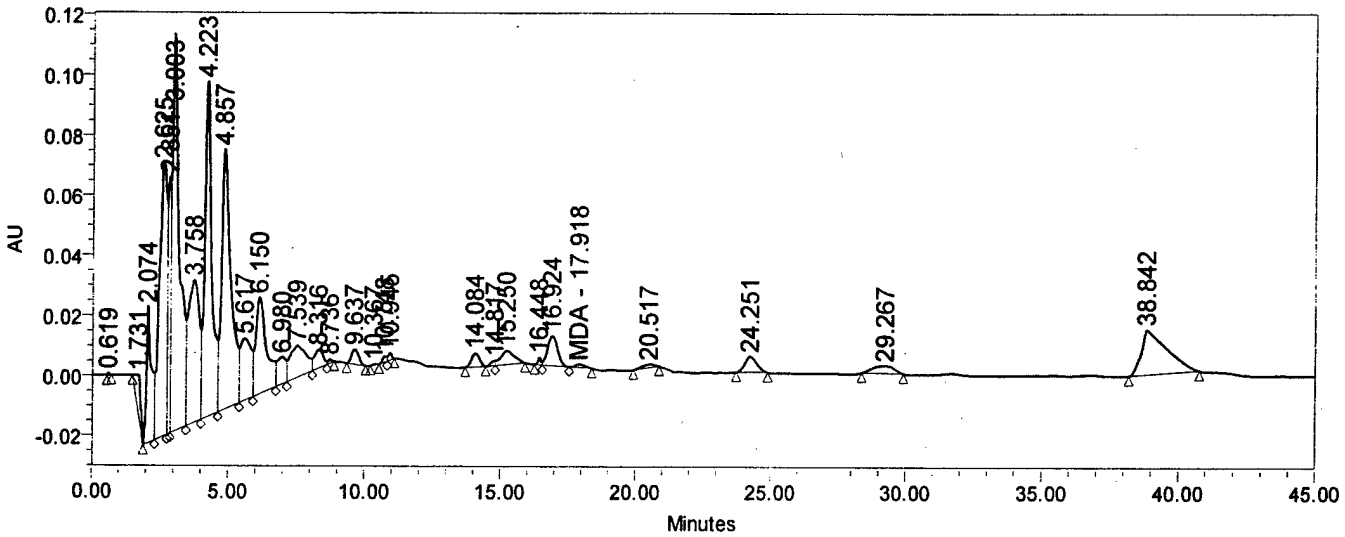
Sample Report

Reported by User: System

Project Name: Vit_D1

SAMPLE INFORMATION

Sample Name:	diet D5	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	2/13/03 11:38:00 AM
Vial:	13	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	2/21/03 1:00:07 PM
Injection Volume:	100.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	486
Sample Set Name:	D5 day7	Proc. Chnl. Descr.:	



	Peak Name	RT	Area	% Area	Height
1		0.619	3443	0.02	803
2		1.731	175568	1.14	13884
3		2.074	570268	3.71	45823
4		2.625	1719988	11.19	91816
5		2.851	578719	3.76	85584
6		3.003	2525682	16.42	132440
7		3.758	1346224	8.75	47393
8		4.223	2042835	13.28	112050
9		4.857	2092671	13.61	87012
10		5.617	564219	3.67	20739
11		6.150	900384	5.86	32228
12		6.980	205765	1.34	9177
13		7.539	445492	2.90	10658

001--433

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
14		8.316	135665	0.88	6088
15		8.736	12938	0.08	1293
16		9.637	93704	0.61	4989
17		10.367	1933	0.01	175
18		10.748	20050	0.13	1998
19		10.945	21779	0.14	2485
20		14.084	95528	0.62	4533
21		14.817	19585	0.13	1680
22		15.250	165908	1.08	4638
23		16.448	20525	0.13	2559
24		16.924	259283	1.69	9965
25	MDA	17.918	27597	0.18	1078
26		20.517	37858	0.25	1170
27		24.251	163323	1.06	5291
28		29.267	143430	0.93	2577
29		38.842	987122	6.42	14869

C01--434

Figure 5: HPLC Chromatograms of D5 in Cornstarch, Week 3

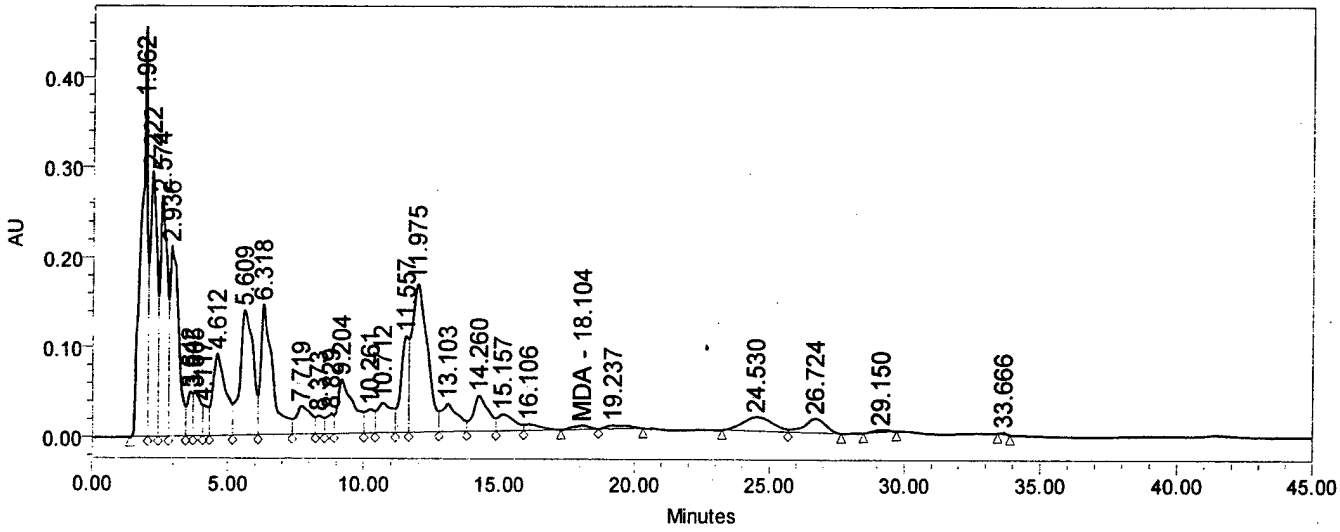
Sample Report

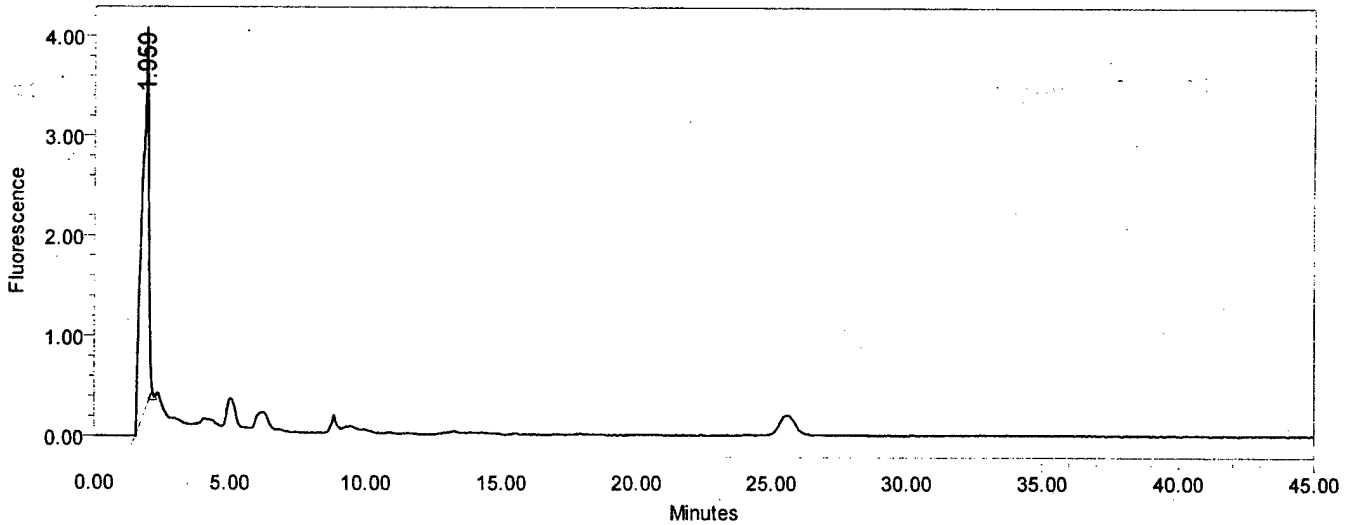
Reported by User: System

Project Name: Vit_D1

SAMPLE INFORMATION

Sample Name:	Rm.temp.Wk3	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	3/18/03 3:02:23 PM
Vial:	9	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	3/18/03 3:47:36 PM, 3/18/03 3:47:37 PM
Injection Volume:	200.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	474 Ch1, 486
Sample Set Name:	Vitamin D5 in corn starch	Proc. Chnl. Descr.:	





	Peak Name	RT	Area	% Area	Height
1		1.959	63115	100.00	3798

Sample Report

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
2		1.962	6672987	11.60	450656
3		2.222	5378922	9.35	295481
4		2.574	4883577	8.49	268737
5		2.936	4820304	8.38	212686
6		3.642	693825	1.21	48950
7		3.805	894596	1.56	48269
8		4.117	489588	0.85	34112
9		4.612	2967837	5.16	91174
10		5.609	4685079	8.14	139461
11		6.318	4409689	7.67	145781
12		7.719	1229572	2.14	31293
13		8.373	384618	0.67	20368
14		8.829	417953	0.73	22546
15		9.204	2404660	4.18	59800
16		10.261	623362	1.08	26352
17		10.712	1260778	2.19	32987
18		11.557	2042116	3.55	107058
19		11.975	6612876	11.50	165113
20		13.103	1346385	2.34	30989
21		14.260	1559796	2.71	39184
22		15.157	810794	1.41	18125
23		16.106	284827	0.50	6732
24	MDA	18.000			
25	MDA	18.104	194350	0.34	4274
26		19.237	233504	0.41	3755
27		24.530	1218685	2.12	15662
28		26.724	878785	1.53	15880
29		29.150	100344	0.17	2638
30		33.666	27354	0.05	1981

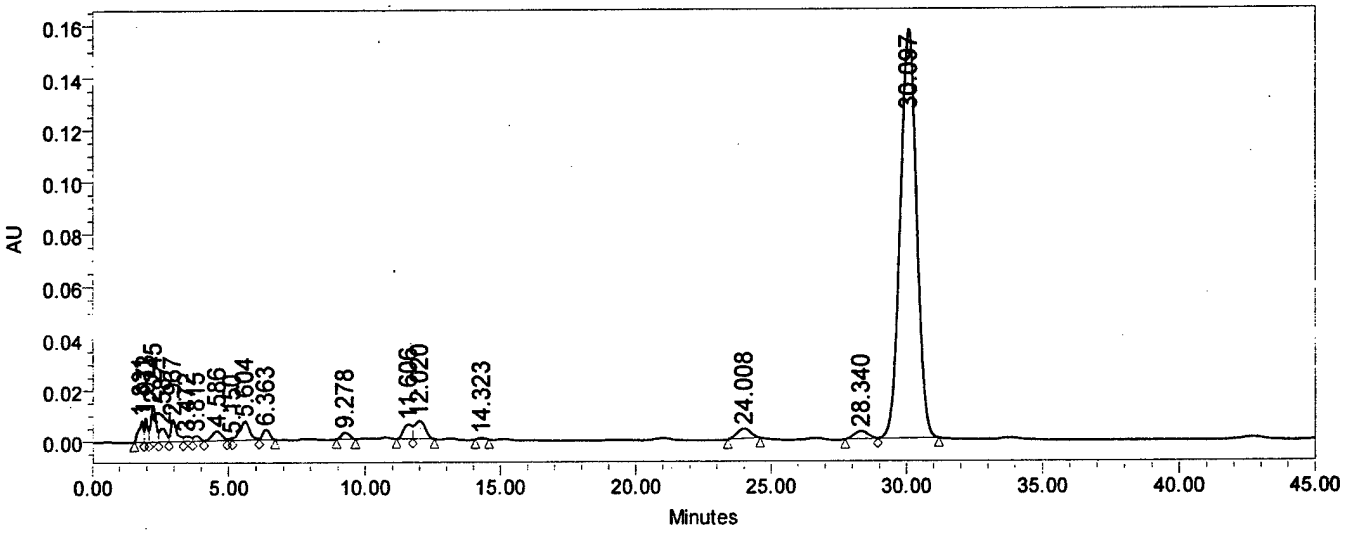
Sample Report

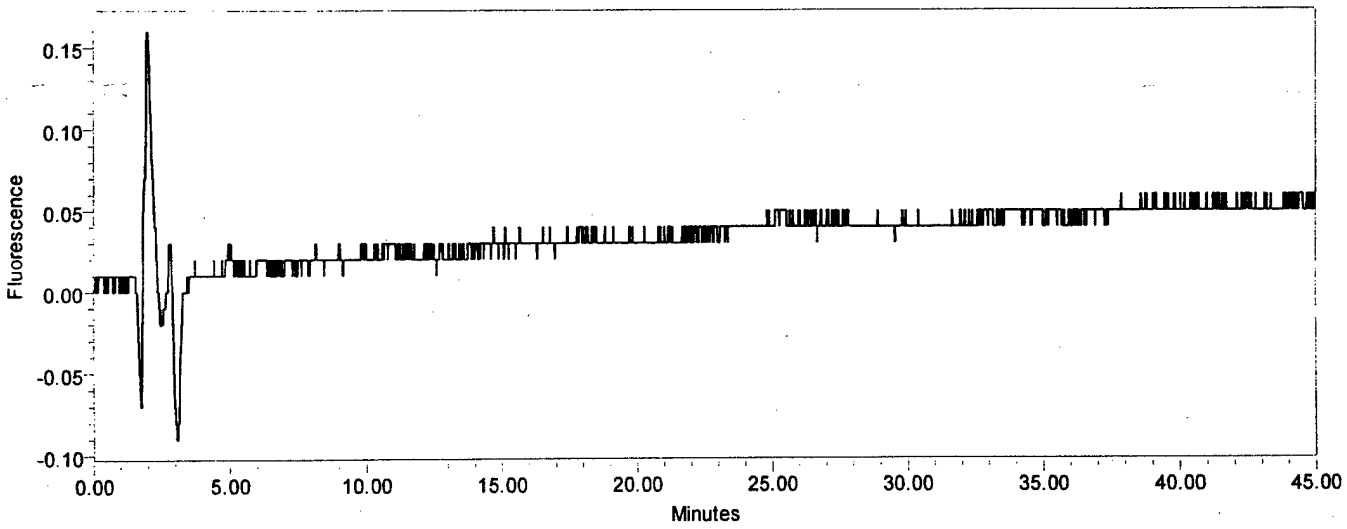
Reported by User: System

Project Name: Vit_D1

SAMPLE INFORMATION

Sample Name:	D5downscale	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	3/19/03 2:09:41 PM
Vial:	9	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	3/19/03 2:54:56 PM
Injection Volume:	100.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	474 Ch1, 486
Sample Set Name:	Chromatographs Mar192003	Proc. Chnl. Descr.:	





	Peak Name	RT	Area	% Area	Height
1		1.831	94631	1.14	8411

Sample Report

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
2		1.973	71621	0.86	9539
3		2.245	188970	2.28	13972
4		2.587	95598	1.15	5401
5		2.967	131878	1.59	8667
6		3.472	36137	0.44	2112
7		3.815	35202	0.42	2109
8		4.586	96898	1.17	3779
9		5.150	9222	0.11	984
10		5.604	189234	2.28	7411
11		6.363	71855	0.87	3968
12		9.278	53780	0.65	2528
13		11.606	126509	1.53	5653
14		12.020	195168	2.36	7112
15		14.323	10012	0.12	544
16	MDA	18.000			
17	MDA	18.000			
18		24.008	137589	1.66	3930
19		28.340	111452	1.35	3051
20		30.097	6628595	80.01	157369

7.4. Placebo Information

No placebo will be used.

7.5. Labeling

PROPOSED CLINICAL TRIAL MATERIAL LABELING – D5 IND

CAUTION: New Drug – Limited by Federal Law (United States) to Investigational Use Only

IND #: 56,509 Drug: 1α-Hydroxyvitamin D_5

Patient ID: _ _ _ Patient Initials: _ _ _

Formulation Date: __/__/___ By: _____

Store at Room Temperature: 59 °F to 77 °F (15 °C to 25 °C).

Dosing Instructions: Take one capsule daily as directed.

Contents: 9 Capsules

Do not discard bottle or any remaining capsules. Bring bottle with you to your next clinic visit.

001--441

8. Pharmacology and Toxicology Data

8.1. Pharmacology and Drug Distribution

8.1.1. Determination of 1α-Hydroxy-vitamin D5 in Mouse Serum

Balb/c female mice were treated with a single intragastric administration of 10 μg/kg BW of 1α(OH)D5. Three animals were sacrificed at each time point of 1, 2, 4,16 and 24 hours after the treatment. Blood was collected, serum was prepared and saved in the freezer at –20 °C until analyzed using LC-MS procedures on a LC-MS Hewlett/Packard Series 1100 MSD, Phenomenex Hypersil C18, 5 micron column, 150x3.20 mm.

Standard curve: 1α-Hydroxy-vitamin D5 was prepared in series concentrations from stock solution (1 mg/ml in methanol) in methanol/water (50:50 v/v) with an internal standard (MW 286.42). 10 μL of each solution was injected for LC-MS (Agilent MSD) analysis. Each solution was analyzed three times at the same condition. The standard curve was obtained by plotting the average peak area ratios of 1α-Hydroxy-vitamin D5 over the internal standard against the corresponding weight of 1α-Hydroxy-vitamin D5 injected.

Serum samples: 50 μL of each serum sample was combined with 100 μL acetonitrile, votexed, then centrifuged at 10,000 rpm for 15 min. The same amount of the internal standard was added to each of the supernatant. 10 μL of each mixture was injected for LC-MS analysis at the same condition.

Table 5. Standard Curve Data

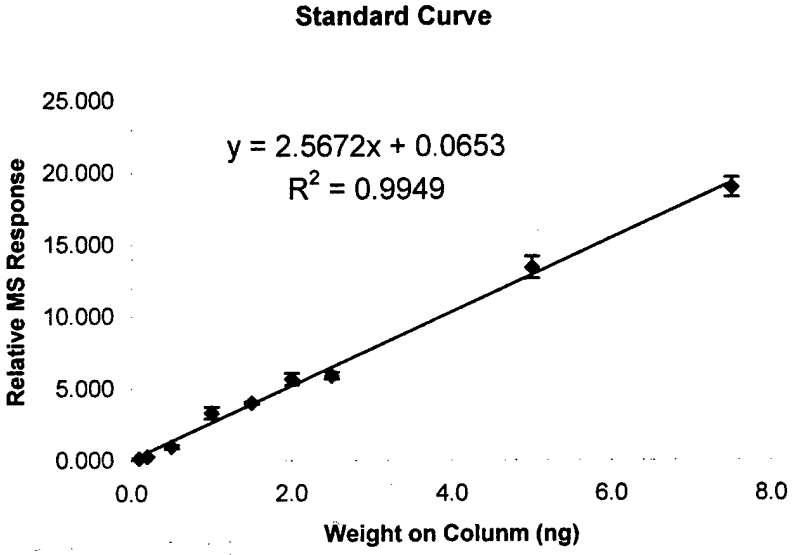
Weight Injected (ng)	Average Peak Area Ratios	Standard Deviation
0.1	0.158	0.016
0.2	0.293	0.012
0.5	0.969	0.141
1.0	3.321	0.407
1.5	4.017	0.085
2.0	5.657	0.408
2.5	5.900	0.235
5.0	13.414	0.762
7.5	18.973	0.693

001--442

Table 6. Serum Data

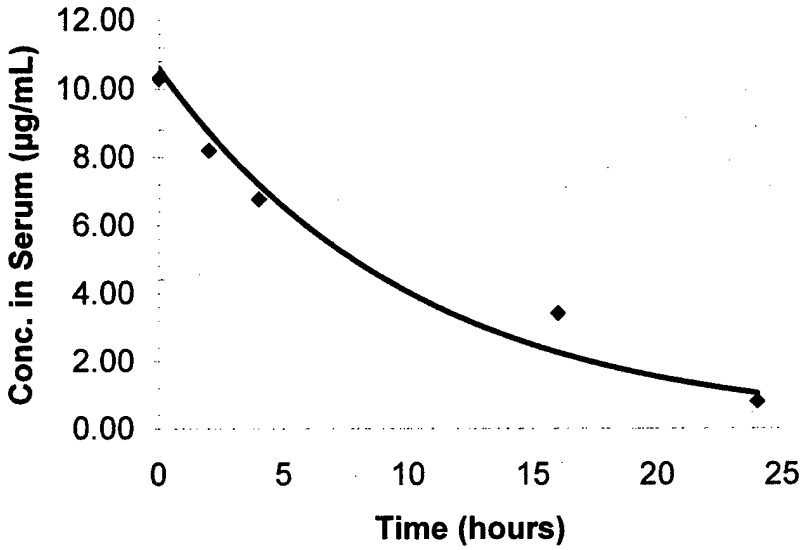
Time (hr.)	Average Concentration in Serum (ng/ml)
0	10.29
2	8.21
4	6.78
16	3.42
24	0.81

Figure 6: Standard Curve



001--443

Figure 7: Average Concentration of D5 in Serum



001--444

8.2. Toxicology: Integrated Summary

The drug, 1α-Hydroxyvitamin D_5, is a cancer chemotherapeutic agent being developed for the treatment of breast cancer. Nonclinical toxicology studies have been conducted in rats and dogs for 28 days. The following is a brief integrated summary of the findings from these two studies. Individual summaries of each study follow in the next section.

The drug was administered to rats and dogs for 28 days. All effects seen in these studies were either directly or indirectly related to the effects of hypervitaminosis from the Vitamin D based drug or possibly its metabolites. The primary effects seems to related to an alteration in calcium metabolism. In rats, beginning at 2.5 μg/kg/day through the highest dose of 10 μg/kg/day, minimal to mild mineralization of different sites in the kidney, basophillic tubules and dilatation of the renal tubules was identified histologically and was still present at the end of a 28 day recovery period. This was supported by in life observations of increased calcium levels (5 μg/kg/day and above) and increased phosphorus levels (10 μg/kg/day). Degeneration of the sternal cartilage was seen as well. Thus, a clear no-effect level was not identified.

Similar effects were seen in the dog, although they were more pronounced due to the higher dose levels employed (up to 90 μg/kg/day). Doses of 30 μg/kg/day were lethal to dogs, with the time to mortality being dose related. Clinical signs were pronounced and tended to reflect the adverse effects of the drug. No clinical signs or body weight effects were seen at 5 μg/kg/day. Reductions in red cell parameters reflected the debilitated condition of the dogs at 30 μg/kg/day and above. Calcium levels were decreased as in the rat study, but phosphorus levels were decreased. Increases in urea nitrogen, cholesterol and triglyceride levels may have reflected the clinical condition of the animals, although the urea nitrogen level increase may be due to the effect on the kidney. Mineralization effects were seen histologically in the kidney and stomach, along with renal tubular dilitation and basophillic tubules. Thymic atrophy was likely due to the stress on the dogs. Hypertrophy/hyperplasia was the thyroid parafollicular cells was also noted. As in the rat study, a clear no-effect level was not identified.

In both studies, the dose of 5 μg/kg/day produced minimal effects that were attributed to the effects of the drug on calcium metabolism. While these effects appear to be nonreversible based on the rat study, the clinical consequences of increased calcium levels could be monitored.

8.2.1. Individual Study Summaries

8.2.1.1. 28-Day Toxicity Study in Rats

The test material, 1α-Hydroxyvitamin D_5, was administered by oral gavage to three groups of 10 male and 10 female Sprague-Dawley rats at doses of 2.5, 5, or 10 μg/kg/day for 28 consecutive days. An additional 10 rats/sex/group were treated with the vehicle only (ethanol/corn oil). In addition, 10 rats/sex were placed into the vehicle and high dose groups and were used for a two-week recovery period. Parameters evaluated included mortality, clinical signs, body weight, food consumption, hematology, clinical chemistry, urinalysis, ophthalmology and gross and microscopic pathology.

There was no mortality during the study, and clinical observations did not identify any treatment related effects. Body weight and food consumption data were unremarkable. Hematological data showed no alterations attributed to treatment, although occasional and sporadic statistically significant changes were noted that showed no dose related trends. Clinical chemistry data showed no effects at the lowest dose of 2.5 μg/kg/day. Increased calcium levels were noted for the 5 (females) and 10 μg/kg/day (both sexes) and increased inorganic phosphorus values were noted at 10 μg/kg/day. These findings were noted at the end of the treatment period, but not at the end of the recovery period, indicating a reversibility of these effects. Ophthalmological examinations were unremarkable. Organ weight data collected at necropsy showed no effects that could conclusively be attributed to treatment, and necropsy findings were unremarkable except for changes normally seen in rats of this age and strain. Histopathological evaluation of tissues showed treatment related effects in the kidney consisting of minimal to mild mineralization (in the medulla, pelvic epithelium and corticomedullary junction), basophillic tubules and dilatation of the renal tubules. These lesions were seen to some extent in all dose groups (except for the low dose males), the incidence appeared to be dose related, and the lesions were still present at the end of the recovery period. Furthermore, degeneration of the sternal cartilage was seen in all dose groups at the end of the treatment period, but was not present at the end of the recovery period. Based on these data, a NOEL was not determined in this study.

8.2.1.2. 28-Day Toxicity Study in Dogs

The test material, 1α-Hydroxyvitamin D_5 was administered by oral gavage to three groups of three male and three female of Beagle dogs of at doses of 10, 30, or 90 μg/kg/day for 28 consecutive days. A fourth group of similar size received the ethanol/corn oil vehicle. In addition, two dogs/sex were placed into the control and highest dose groups and were originally scheduled to be used for

a two week recovery period. Due to marked toxicity that included mortality at the 90 μg/kg/day group, the study design was changed such that 3 males and 3 females received 0, 10 and 30 μg/kg/day, 5 males and 3 females received 45 μg/kg/day, and 2 males and 2 females received 5 μg/kg/day. The recovery groups were eliminated. Parameters evaluated included mortality, clinical signs, physical examination, electrocardiography, ophthalmology, body weight, food consumption, hematology, clinical chemistry, urinalysis, and gross pathology. Histopathological evaluation of tissues was performed on control dogs and primarily those treated at 5 and 10 μg/kg/day.

Eight animals did not survive treatment (3 males and 2 females at 90 (subsequently reduced to 45 μg/kg/day, and 2 males and 1 female at 30 μg/kg/day). The time to mortality or premature sacrifice was dose dependent (after 5-7 doses at 90 μg/kg/day, 14-18 doses at 45 μg/kg/day, and 23-27 doses at 30 μg/kg/day. Clinical signs seen at 10 μg/kg/day and above included emaciation, emesis, bloody salivation, dehydration, cold skin, hypoactivity, lacrimation and reduced body temperature. Signs at 10 μg/kg/day were minimal, and no clinical signs were seen at 5 μg/kg/day. Reduction in body weight gain or body weight loss (along with a reduction in food consumption) was seen at all doses except 5 μg/kg/day. Hematological studies indicated increased values for red cell parameters (RBC, HGB and HCT) that were likely due to dehydration. Clinical chemistry data indicated increased calcium levels and decreased phosphorus levels at all dose in a dose-related fashion. At 30 μg/kg/day and higher, urea nitrogen, cholesterol and triglyceride levels were increased (urea nitrogen was increased in the 10 μg/kg/day males. At necropsy, organ weight data was influenced by the significant body weight losses, but decreased weights for the thymus were attributed to drug treatment and were likely related to stress. Histopathological evaluation of tissues indicated effects in the kidney (cortical mineralization, tubular dilatation and basophillic tubules), stomach (mid-mucosal pyloric mineralization), thymic atrophy, and hypertrophy/hyperplasia of the thyroid parafollicular cells.

8.4. Toxicology – GLP Certification

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1α-HYDROXYVITAMIN D_5 IN RATS

GLP COMPLIANCE STATEMENT

This study was conducted in accordance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations as set forth in the *Code of Federal Regulations* (21 CFR Part 58). The identity, purity and stability of the bulk test article were the responsibility of the Sponsor. The vehicle (corn oil) was a purchased product and, as such, was characterized by a Certificate of Analysis provided by the vendor. The study raw data have been reviewed by the Study Director, who certifies that the information contained in this report accurately reflects and is supported by the data.

William D. Johnson 12-6-00

William D. Johnson, Ph.D., D.A.B.T. Date
Study Director
Life Sciences Operation

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1α-HYDROXYVITAMIN D_5 IN BEAGLE DOGS

GLP COMPLIANCE STATEMENT

This study was conducted in accordance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations as set forth in the *Code of Federal Regulations* (21 CFR Part 58), with the exceptions that 1) stability of the dosing formulations was not determined for the low end of the dose concentration range (*i.e.*, concentrations analyzed were 10, 30 and 90 µg/ml; concentrations dosed were 5, 10, 30, 45 and 90 µg/ml) and 2) the dosing formulation stability determinations did not always cover the entire period between preparation and completion of use (*i.e.*, stability was determined for an 8-day period; 8-12 days elapsed between preparation and end of use). The identity, purity and stability of the bulk test article were the responsibility of the Sponsor, and a copy of the Certificate of Analysis provided is included in Appendix B of the report. Dosing formulations of the test article were analyzed to verify concentration, homogeneity and stability. The vehicle (corn oil) was a purchased product and, as such, was characterized by a Certificate of Analysis (Appendix B) provided by the vendor.

The study raw data have been reviewed by the Study Director, who certifies that the information contained in this report accurately reflects and is supported by the data.

William D. Johnson 10-3-01

William D. Johnson, Ph.D., D.A.B.T. Date
Study Director
Life Sciences Operation

9. Previous Human Experience

There is no previous human experience with the drug.

10. Claim for categorical exclusion from or submission of an environmental assessment.

The sponsor believes this IND meets the exclusion categories listed in 21 CFR 25.31, and therefore requests a categorical exclusion from the requirement for an environmental assessment.

001--559

APPENDIX

Publications:

Mehta R.G. and Mehta R.R. Vitamin D and Cancer J. Nutr. Biochem. 13: 252-264, 2002

Mehta R.G., Hussain E.A., Mehta R.R., Das Gupta T.K. Chemoprevention of mammary carcinogenesis by 1α-Hydroxyvitamin D5, a synthetic analog of vitamin D. Mutation Res. 523: 253-264, 2003

Hussain E.A., Mehta R.R., Ray R., Das Gupta T.K. and Mehta R.G. efficacy and mechanism of action of 1α-hydroxy-24-ethyl-cholecalciferol (11(OH)D5) in breast cancer prevention and therapy. Recent Results in Cancer Res. 164:393-411, 2003.

Vasu P., Graves JG. Mehta R.R. Effect of vitamin D analog (1 alpha hydroxy D5) immunoconjugated to Her-2 antibody on breast cancer. Int. J. Cancer (In Press).

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Chemoprevention of mammary carcinogenesis by 1α-hydroxyvitamin D_5, a synthetic analog of Vitamin D

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Received 1 April 2002; received in revised form 10 August 2002; accepted 3 September 2002

Abstract

Numerous analogs of Vitamin D have been synthesized in recent years with the hope of generating a compound that retains the anticarcinogenic activity of Vitamin D without causing any toxicity. We synthesized such an analog, 1α-hydroxy-24-ethylcholecalciferol [1α-hydroxyvitamin D_5 or 1α(OH)D_5], and showed that it was tolerated by rats and mice at a much higher dose than 1α,25 dihydroxy cholecalciferol [1α,25$(OH)_2D_3$]. This property makes it a prime candidate for chemoprevention studies. In the mouse mammary gland organ culture (MMOC), 1α(OH)D_5 inhibited carcinogen-induced development of both mammary alveolar and ductal lesions. In vivo carcinogenesis study showed statistically significant reduction of tumor incidence and multiplicity in *N*-methyl-*N*-nitrosourea (MNU)-treated rats that were fed 25–50 μg 1α(OH)D_5/kg diet. There were no adverse effects on plasma calcium concentrations. In order to determine if the effect of 1α(OH)D_5 would be selective in suppressing proliferation of transformed cells, its effects on cell growth and proliferation were compared between BT474 (cancer) and MCF12F (non-tumorigenic) human breast epithelial cells. Results showed that 1α(OH)D_5 induced apoptosis and cell cycle G1 phase arrest in BT474 breast cancer cells without having any effects on proliferation of the MCF12F cells. In addition, in MMOC it had no growth inhibitory effects on normal epithelial cell proliferation in the absence of carcinogen. Similarly, non-tumorigenic human breast epithelial cells in explant culture did not respond to 1α(OH)D_5, whereas treatment with 1α(OH)D_5 induced cell death in the explants of cancer tissue. These results collectively indicate that 1α(OH)D_5 selectively induced apoptosis only in transformed cells but not in normal breast epithelial cells. Interestingly, the growth inhibitory effects of 1α(OH)D_5 were observed in Vitamin D receptor positive (VDR^+) breast cancer cells, but not in highly metastatic VDR^- breast cancer cells, such as MDA-MB-435 and MDA-MB-231, suggesting that 1α(OH)D_5 action may be mediated, in part, by VDR.

Keywords: Vitamin D; Mammary carcinogenesis; Chemoprevention

1. Introduction

Conceptually, chemoprevention of cancer can be defined as an intervention in the carcinogenic process by either a naturally derived or a synthetic compound. An agent that blocks, arrests, or reverses the progression of cancer can be termed a chemopreventive agent [1,2]. In practice, this can best be achieved by the dietary administration of chemical agents, which can enhance the physiological processes that protect the organism against the development of malignancy. Current understanding of progression of a normal

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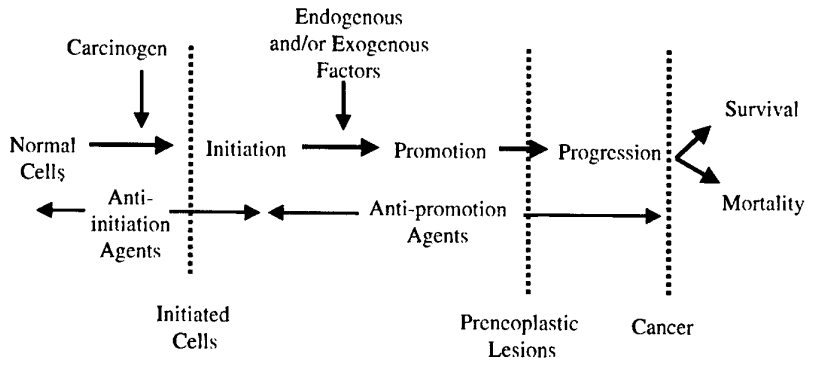

Fig. 1. Schematic diagram to show stages in mammary carcinogenesis and potential points of intervention by chemopreventive agents.

cell to a transformed cancer cell is summarized in Fig. 1. Under experimental conditions, a normal cell could be transformed to an initiated cell in response to carcinogenic or mutagenic stimuli. Although the initiated cells have the potential to develop into malignant cancer, they may or may not form a tumor depending upon exposure to exogenous and/or endogenous factors. In the absence of growth arrest stimuli, the initiated cell can advance to a preneoplastic stage leading progressively to malignancy. The chemopreventive agents that suppress the early events in transformation, such as preventing the mutagenic action of chemicals or other factors, are referred to as anti-initiation agents. On the other hand, chemicals that prevent further progression of initiated cells into transformed ones are termed anti-promotional agents [3,4]. Numerous classes of chemopreventive agents have been reported in the literature, including retinoids, deltanoids, cyclooxygenase inhibitors, inhibitors of polyamine and prostaglandin biosynthesis, lignans, calcium channel blockers, anti oxidants, etc. [5–7]. In this report, we have summarized the chemopreventive properties of a newly evaluated Vitamin D analog, 1-α-hydroxy-24-ethyl-cholecalciferol [1α(OH)D_5].

It has been well established that the active metabolite of Vitamin D, 1α,25-dihydroxyvitamin D_3, [1,25$(OH)_2D_3$] is a steroid hormone and it exhibits potent cell-differentiating properties in leukemia cells as well as other cancer cells of epithelial origin [8,9]. The antiproliferative and differentiation-inducing effects of 1,25$(OH)_2D_3$ could be of clinical significance in prevention or treatment of cancer of several target organs [10]. However, one major limitation in its clinical application is the fact that the efficacious concentrations of 1α,25$(OH)_2D_3$ are cytotoxic [11]. The effective growth inhibitory concentration of 1α,25$(OH)_2D_3$ induces dangerously high levels of serum calcium resulting in loss of body weight and soft tissue calcification, which could be lethal [12]. This has resulted in generation of several non-toxic but antiproliferative synthetic analogs of the Vitamin D molecule for the prevention and treatment of cancer. Some of these analogs have been successfully evaluated for their ability to suppress cancer cell growth in culture as well as in vivo models [13].

Typically, the structure of Vitamin D is divided into four parts (Fig. 2): ring A, open ring B, ring CD, and the side chain. Modifications can be made at all four sites, but the alteration of the ring CD is not common due to its rigid structure. Most alterations have been made at the open side chain. Nearly 800 analogs of Vitamin D have been synthesized so far, and about 300 of them have been evaluated in in vitro and in vivo experimental models [14,15]. Historically, a comparison of the toxicological profile of the Vitamin D series of compounds, including D_2–D_6, had suggested that D_5 was the least toxic of the D series of compounds [16]. In order to generate an effective but non-calcemic and non-toxic Vitamin D analog, we synthesized 1α(OH)D_5 [17]. The structure of 1α(OH)D_5 is shown in Fig. 2.

Vitamin D hormone mediates its action by both genomic and non-genomic pathways. The genomic

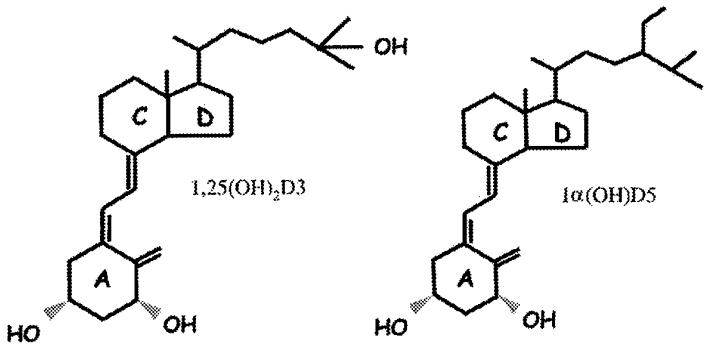

Fig. 2. Structural representation of $1,25(OH)_2D_3$ and its analog $1\alpha(OH)D_5$.

pathway involves its association with high-affinity specific Vitamin D receptor (VDR) that belongs to the steroid receptor superfamily of ligand-activated transcription factors [18–20]. This is consistent with the well-known mode of action of the steroid hormones. The VDR has been identified in a variety of tissues such as breast, prostate, liver, fibroblasts, colon, and lungs [21], in addition to the previously known target organs that included intestine, kidney, and bone.

The VDR mRNA is about 4.6 kb, which translates to a 50-kd protein in humans. The VDR content ranges from 400 to 27,000 copies per cell, yielding 10–100 fmoles/mg of total protein. In order for VDR to function, it needs to bind specific DNA sequences and interact with Vitamin D response elements (VDRE) [22]. The natural metabolite $1\alpha,25(OH)_2D_3$ transactivates VDRE in VDR^+ cells but fails to show interaction in VDR^- cells. Hence, Vitamin D analogs that are able to transactivate VDR–VDRE are mainly mediating their action via genomic pathways. Non-genomic Vitamin D actions have been studied mostly in relation to calcium and phosphorus metabolism, and to a lesser extent with respect to chemoprevention. The rapid responses involve a putative membrane receptor of Vitamin D that signals to modulate calcium channel activity in a cell. This may lead to exocytosis of calcium-bearing vesicles from lysosomes. The non-genomic pathway for Vitamin D action has been extensively reviewed elsewhere [23,24]. For this article, we have listed the chemopreventive properties and possible mode of action of $1\alpha(OH)D_5$.

2. Materials and methods

2.1. Cell lines

We purchased from the American Type Culture Collection (ATCC), Bethesda, MD and maintained in our laboratory according to the ATCC recommendations the following cell lines: (1) the non-tumorigenic, estrogen receptor-negative (ER^-), progesterone receptor-negative (PgR^-), and low VDR breast epithelial cell line MCF12F; (2) ER^+, PgR^+, and VDR^+ breast cancer cell lines BT474 and MCF7; and (3) ER^-, PR^-, and VDR^- breast cancer cell lines MDA-MB-231 and MDA-MB-435.

2.2. Mouse mammary gland organ culture (MMOC)

The detailed procedures for culturing mammary glands from Balb/c mice have been previously reported in the literature [17,25] and outlined in Fig. 3. Briefly, thoracic pairs of mammary glands from Balb/c mice are maintained in serum-free Waymouth's MB752/1 medium under 95% O_2 and 5% CO_2 at 37 °C. The glands respond to growth-promoting hormones insulin, prolactin, aldosterone, and hydrocortisone and differentiate into distinct alveolar structures. Exposure of glands to 7,12-dimethylbenz(a)anthracene (DMBA) for 24 h on day 3 of culture results in the development of precancerous mammary alveolar lesions (MAL). If the growth-promoting medium contains estrogen and progesterone instead of aldosterone and hydrocortisone, the

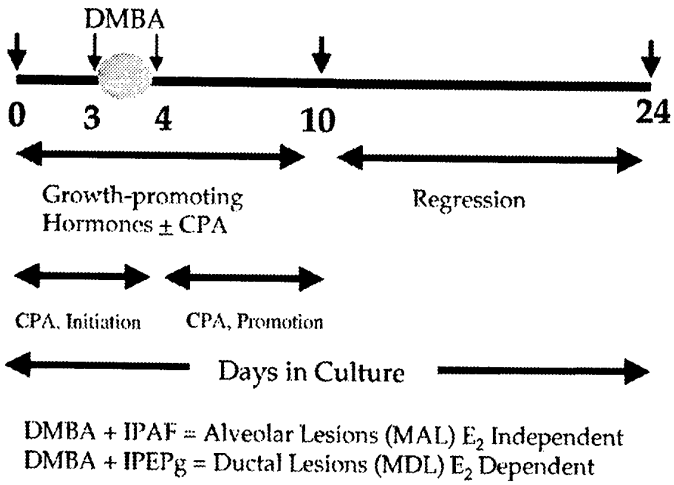

Fig. 3. Experimental design for chemoprevention in mouse mammary gland organ culture (MMOC). DMBA: 7,12-dimethylbenz(a)anthracene, CPA: chemopreventive agent, IPAF: insulin + prolactin + aldosterone + hydrocortisone, IPEPg: insulin + prolactin + estradiol + progesterone, MAL: mammary alveolar lesions, MDL: mammary ductal lesions.

glands develop mammary ductal lesions (MDL) with DMBA treatment [26]. We performed a dose response study to compare the effects of $1\alpha(OH)D_5$ on MAL and MDL. Mammary lesions developed in the absence of $1\alpha(OH)D_5$ served as controls. Additionally, we determined the effects of $1\alpha(OH)D_5$ on normal mammary glands, where the glands were incubated with growth-promoting hormones and 1 μM $1\alpha(OH)D_5$ for 6 days without DMBA treatment. The glands from these MMOC experiments were fixed, stained, and analyzed for morphological characteristics and cell growth and compared with the appropriate controls.

2.3. Cell cycle analysis by flow cytometry

To determine cell cycle, we used flow cytometric analysis as described by Vindeløv et al. [27]. Breast epithelial non-tumorigenic and cancer cells were detached by trypsinization and were harvested. The cells were washed twice with PBS and pelleted. The pellet was resuspended and fixed in 85% ice-cold ethanol. After fixing, the cells were centrifuged and resuspended in citrate buffer and then incubated with NP-40, trypsin, and sperminc for 15 min. This was followed by incubation with trypsin inhibitor and RNAase A. The cells were then stained with 0.04% propidium iodide solution. Approximately 10,000 cells were analyzed for DNA content using a Beckman-Coulter EPICS Elite ESP flow cytometer. Multicycle analysis software was used to determine the percentage of cells in various stages of cell cycle. Each experiment was repeated twice and student's *t*-test was used to assess differences.

2.4. Apoptosis

Programmed cell death was evaluated using acridine orange staining. Briefly, a 50 μl suspension of breast epithelial cells was stained with 2 μl of acridine orange/ethidium bromide solution (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS). Cells were layered on a glass slide and examined under a fluorescent microscope with a 40× objective lens using a flourescein filter. Approximately 100 cells were counted on each slide to assess the proportion of cells undergoing apoptosis.

2.5. Mammary carcinogenesis

The procedure for induction of mammary adenocarcinomas by *N*-methyl-*N*-nitrosourea (MNU) in Sprague–Dawley female rats has been described in detail previously [28] and is illustrated in Fig. 4. Briefly, 100-day-old female Sprague–Dawley rats

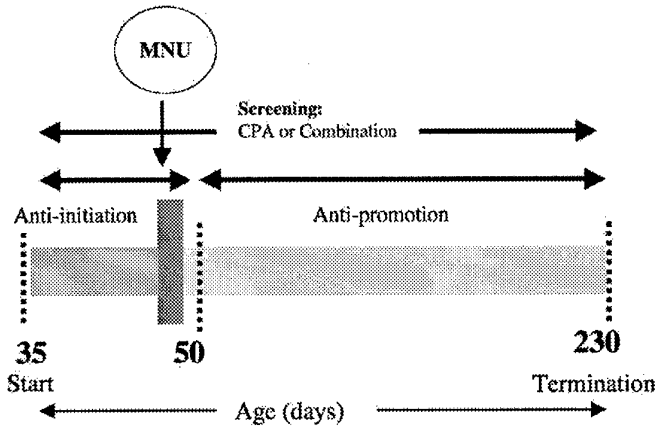

Fig. 4. Schematic diagram to show in vivo model of chemoprevention in *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in Sprague–Dawley rats. CPA: chemopreventive agent.

were injected subcutaneously with 50 mg/kg MNU prepared in acidified saline. Animals received either placebo or $1\alpha(OH)D_5$ supplemented as 25 or 50 μg/kg diet. Animals were sacrificed after 230 days of treatment. Mammary tumors were identified by palpation as well as necroscopy. Results were reported as effects of $1\alpha(OH)D_5$ on the incidence, multiplicity, and latency of tumor development, and data were subjected to appropriate statistical analyses.

2.6. Effects of $1\alpha(OH)D_5$ on normal and malignant breast tissue

Breast tissues were obtained from women undergoing mastectomy or lumpectomy. Explants were maintained in MEME medium, containing 5% stripped fetal bovine serum. The effects of 1 μM $1\alpha(OH)D_5$ were determined on these tissues by evaluating cell morphology, apoptosis, and expression of Ki 67. The effects of $1\alpha(OH)D_5$ on cell morphology and Ki 67 were compared between the normal and adjacent cancer tissue from the same patient.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Instat® 3.0 software. All MMOC as well as MNU-induced carcinogenesis data were evaluated using χ^2 analysis. Cell viability, apoptosis, and cell cycle results were assessed using two-tailed student's *t*-test with type I error set at 0.05. Serum calcium and phosphorus data were tested with student's *t*-test as well. All in vitro experiments were performed in duplicates and repeated twice.

3. Results and discussion

3.1. Synthesis and toxicity of $1\alpha(OH)D_5$

Nearly 300 analogs of $1,25(OH)_2D_3$ have been evaluated in various experimental systems in the hope of generating analogs that are more efficacious with reduced toxicity. Among the analogs evaluated, only a few have shown potent chemopreventive and therapeutic activity. These analogs include EB1089 [29], KH1060 [30], R024-5531 [31], and 22-Oxacalcitriol [32], which are relatively nontoxic at effective concentrations in experimental models. The hexafluoro analog of $1,25(OH)_2D_3$, R024-5531, has no calcemic activity, while other analogs do express dose-related calcemia [33,34]. Since it had been reported previously that Vitamin D_5 is the least toxic series of Vitamin D compounds, we synthesized $1\alpha(OH)D_5$ with the intention of testing its chemopreventive potential. The chemical synthesis of $1\alpha(OH)D_5$ has been previously reported from our laboratory [17].

Since calcemic activity is an obstacle to the development of effective Vitamin D analogs suitable for clinical use, we determined serum calcium and phosphorous concentrations after treating Vitamin D-deficient rats with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$. As reported earlier, male Sprague–Dawley rats (8–10 per group) were fed Vitamin D-deficient diet for 3 weeks, and baseline serum calcium levels were determined. Rats showing <6 mg/dl serum calcium were given $1\alpha(OH)D_5$ for 14 days. Subsequently, serum calcium concentrations were measured. Results showed that $1,25(OH)_2D_3$ significantly ($P < 0.001$) increased serum calcium concentration at a daily dose of 0.042 μg/kg diet, whereas there was no elevation in serum calcium levels among $1\alpha(OH)D_5$-treated animals [17].

A similar experiment was carried out using Vitamin D-sufficient regular diet. Female Sprague–Dawley rats were treated with various concentrations of $1,25\ (OH)_2D_3$ (0.8–12.8 μg/kg diet) and $1\alpha(OH)D_5$ (6.4–50 μg/kg diet) for 2 months. Calcium concentration was increased by $1,25\ (OH)_2D_3$ treatment, while no serum calcium elevation was observed in $1\alpha(OH)D_5$-treated (25 μg/kg diet) animals (Table 1). There was no effect on the final body weight at any dose of $1\alpha(OH)D_5$ used in this study. These results indicate that $1\alpha(OH)D_5$ is considerably less toxic compared to the natural hormone.

More recently, we completed an extensive preclinical toxicity study in both sexes of rats and dogs under good laboratory practice (GLP). Results showed that dogs are relatively more sensitive to the higher dose of $1\alpha(OH)D_5$ than are rats. We concluded from those studies that $1\alpha(OH)D_5$ is calcemic in dogs at concentrations higher than 10 μg/kg diet. The non-calcemic analog R024-5531 shows toxicity in rats without having an effect on serum calcium concentrations. On the other hand, $1\alpha(OH)D_5$ can be tolerated at a higher concentration without other toxicity outcomes.

Table 1
Effects of $1\alpha(OH)D_5$ treatment on serum calcium and phosphorous levels in Sprague–Dawley rats ($n = 10$)

Agent	Dose (μg/kg)	Serum Ca (mg/dl)	Serum P (mg/dl)[a]	BW (% gain)
None		6.3	3.6	100
$1,25(OH)_2D_3$	0.8	7.0	6.4	101
	3.2	7.1	8.0	104
	12.8	7.5*	8.9	70*
$1\alpha(OH)D_5$	6.4	6.3	7.2	99
	12.5	6.2	7.2	97
	25.0	6.5	7.1	98
	50.0	ND	ND	113

* Significantly different from control ($P < 0.05$).
[a] Significance not determined.

Chemoprevention of mammary carcinogenesis by $1\alpha(OH)D_5$: The chemopreventive properties of $1\alpha(OH)D_5$ have been evaluated in two experimental systems in our laboratory. These include MMOC and MNU-induced mammary carcinogenesis in Sprague–Dawley rats. Mouse mammary glands respond to DMBA and develop preneoplastic mammary alveolar as well as ductal lesions in organ culture. As shown in Fig. 3, the efficacy of a potential chemopreventive agent can be assessed in this assay. If the agent is present and effective prior to carcinogen treatment, its effects are considered as anti-initiation, whereas, if it is effective subsequent to carcinogen, then its effect are anti-promotional. Both types of effects can be determined using the MMOC model.

We showed previously that $1\alpha(OH)D_5$ inhibits the development of mammary lesions in a dose-responsive manner [17]. However, it requires 10-fold higher concentration than the effective concentration of $1,25(OH)_2D_3$. The most effective dose of $1,25(OH)_2D_3$ in suppressing >60% incidence of MAL is 10^{-7} M, while $1\alpha(OH)D_5$ is equally effective at 10^{-6} M without showing cytotoxicity. We also evaluated $1\alpha(OH)D_5$ effects in the MDL model [25]. The results are summarized in Fig. 5. We found $1\alpha(OH)D_5$ to be equally effective against alveolar and ductal lesions.

Since most of the effects of Vitamin D are mediated through VDR, we determined VDR induction by $1\alpha(OH)D_5$ in MMOC as well as in breast cancer cell lines [17]. There was a significant increase in the expression of VDR in the epithelial cells of MMOC as determined by immunocytochemistry. Additionally, $1\alpha(OH)D_5$ also upregulated the expression of TGFβ in the epithelial cells of MMOC [15].

Based on these results, it was reasonable to expect chemopreventive activity of $1\alpha(OH)D_5$ in an in vivo model. Prior to conducting in vivo carcinogenesis studies, a dose tolerance study was conducted in Sprague–Dawley rats. Animals were provided with increasing concentrations of $1\alpha(OH)D_5$, ranging from

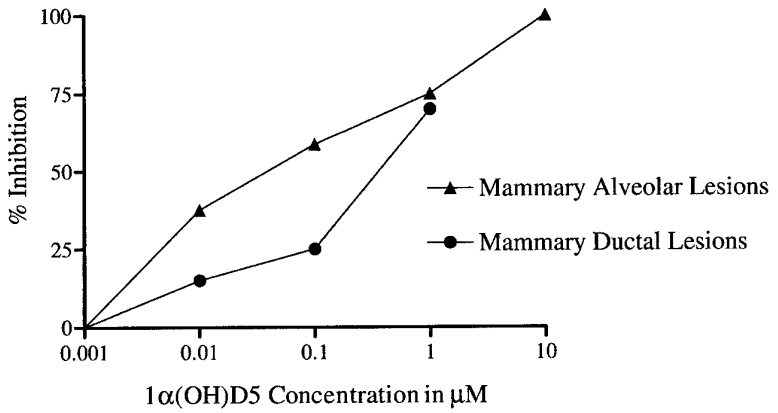

Fig. 5. Effect of $1\alpha(OH)D_5$ on mouse mammary organ culture (MMOC). The glands were incubated with 1 μM $1\alpha(OH)D_5$ for 10 days. The glands were fixed and evaluated for inhibition of preneoplastic lesions in relation to control. Fifteen glands were used per group. A difference in inhibition of greater than 60% was considered significant ($P < 0.05$, χ^2). Data shows significant inhibition of preneoplastic MAL and MDL with $1\alpha(OH)D_5$ treatment.

1 to 100 μg/kg diet for 6 weeks. The animals did not show any adverse effects at any concentration of $1\alpha(OH)D_5$, while the natural hormone was toxic at 3.5 μg/kg diet.

For the MNU-induced mammary carcinogenesis studies, animals were fed $1\alpha(OH)D_5$ at 25 and 50 μg/kg diet for 3 months. The experimental diet was given to the animals 1 week prior to the carcinogen treatment and continued until the end of the study. Results are shown in Table 2. The results indicated a dose-dependent suppression of tumor incidence by $1\alpha(OH)D_5$. This was accompanied by a reduction in tumor multiplicity and an increase in tumor latency [28]. These results are comparable with those of EB1089, R024-5531, and KH1060. The in vivo results as well as the results from MMOC clearly suggest a potential for $1\alpha(OH)D_5$ to be developed as a chemopreventive and therapeutic agent.

Table 2
Chemoprevention of MNU-induced mammary carcinogenesis by $1\alpha(OH)D_5$ in rats

Treatment	Dose (μg/kg)	*n*	Incidence (%)	Multiplicity	Final BW (g)
Control	0	15	80	1.6	228
$1\alpha(OH)D_5$	25	15	53*	1.2	230
$1\alpha(OH)D_5$	50	15	47*	0.8*	226

* Significantly different from control ($P < 0.05$).

3.2. *Selectivity of $1\alpha(OH)D_5$ action for transformed cells*

We compared the growth effects of $1\alpha(OH)D_5$ in various steroid receptor-positive as well as negative breast epithelial cell lines. These cell lines included (1) non-tumorigenic MCF12F breast epithelial cells, (2) ER^+, PgR^+, VDR^+, BT474, and MCF7 cells, and (3) ER^-, PgR^-, and VDR^- highly metastatic MDA-MB-435 and MDA-MB-231 breast cancer cell lines. The results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ were efficacious in suppressing cell proliferation of ER^+, PR^+, and VDR^+ BT474, T47D, ZR75, and MCF7 breast cancer cells. These compounds induced differentiation of ER^-, PgR^-, VDR^+, and BCA-4 cells [35] but did not show any growth effects in MDA-MB-435 and MDA-MB-231 cells. Other researchers have also reported similar results with other Vitamin D analogs [36]. Although our results indicate that the presence of VDR is necessary to potentiate Vitamin D's effect, it does not explain the lack of Vitamin D's effect on MCF12F cells that express low levels of VDR.

In order to examine whether $1\alpha(OH)D_5$ selectively inhibits cell proliferation in transformed cells only, we evaluated the effects of $1\alpha(OH)D_5$ on non-tumorigenic breast epithelial cells and compared them to the effects on BT474 breast cancer cells.

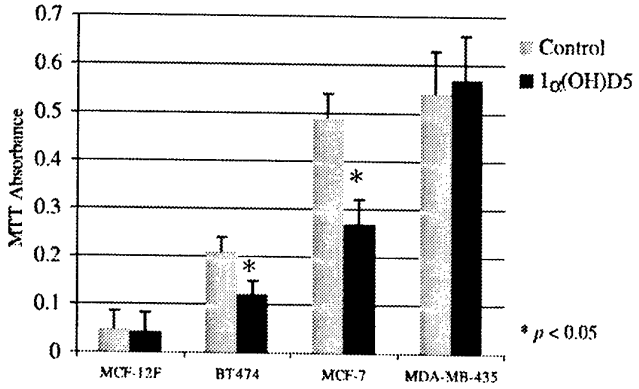

Fig. 6. Effects of $1\alpha(OH)D_5$ on viability of non-tumorigenic and cancer breast epithelial cells. Different cell lines were treated with 1 μM $1\alpha(OH)D_5$ for 2 days and incubated with MTT for 2 h. The cells were lysed and washed prior to reading absorbance at 550 nm. MTT absorbance is proportional to the number of live cells. Each experiment was repeated twice and differences between the mean were assessed using student's *t*-test.

As shown in Fig. 6, incubation of MCF12F breast epithelial cells for 6 days with $1\alpha(OH)D_5$ at 1 μM concentration did not result in suppression of cell proliferation as determined by the MTT absorbance assay. On the other hand, there was a significant inhibition of proliferation in both MCF7 and BT474 cells with $1\alpha(OH)D_5$ treatment. These results suggested that the effect of Vitamin D analog might be selective for transformed cells. The antiproliferative effects of $1\alpha(OH)D_5$ were also evident in in vivo experiments. Xenograft of ER^+, PgR^+, VDR^+, MCF7, ZR75/1, and BT474 cells or ER^-, PgR^-, VDR^+, and BCA-4 cells responded to 12.5 μg $1\alpha(OH)D_5$/kg diet and showed suppressed growth of these cells in athymic mice [35].

To confirm the selectivity of $1\alpha(OH)D_5$ for transformed breast cancer cells, we conducted three separate experiments. In the first experiment, we compared the efficacy of $1\alpha(OH)D_5$ between MCF12F cells with that of MNU-transformed MCF12F ($MCF12F_{MNU}$) cells. The $MCF12F_{MNU}$ cells have recently been established in our laboratory (unpublished data). The $MCF12F_{MNU}$ cells have altered morphology and growth properties as well as different growth factor requirements (Hussain and Mehta, unpublished data). Incubation of MCF12F and $MCF12F_{MNU}$ with 1 μM $1\alpha(OH)D_5$ for 6 days resulted in 50% growth inhibition in $MCF12F_{MNU}$ cells without having any significant effects on MCF12F growth.

In a second study using the MMOC model, the effects of $1\alpha(OH)D_5$ were determined in mammary glands. Mammary glands respond to growth-promoting hormones and develop structurally differentiated alveoli within 6 days in culture. Incubation of glands with 1 μM $1\alpha(OH)D_5$ for 6 days did not affect the growth-promoting effects of insulin, prolactin, aldosterone, hydrocortisone, estrogen, and progesterone (Fig. 7). Contrarily, $1\alpha(OH)D_5$ showed excellent anti-proliferative effects against DMBA-induced MAL and MDL (Fig. 5).

Experiments to determine the selectivity of $1\alpha(OH)D_5$ action against transformed cells were further extended to human tissues. The effects of $1\alpha(OH)D_5$ on the explants derived from normal breast tissues were compared with those of cancer tissue. Breast tissue samples were obtained from women undergoing mastectomy or lumpectomy at the University of Illinois at Chicago Hospital. Tissue explants of tumors and normal adjacent cells were incubated for 72 h in the MEME containing 5% fetal calf serum with or without $1\alpha(OH)D_5$ at 1 μM concentration. Tissue sections were histopathologically evaluated, and Ki 67 expression was determined. Results showed that the histopathology of control and $1\alpha(OH)D_5$-treated normal breast tissue was identical with no difference in apoptosis or Ki 67 expression. On the other hand, the histological sections of the cancer tissue explants showed extensive apoptosis within the tissue with

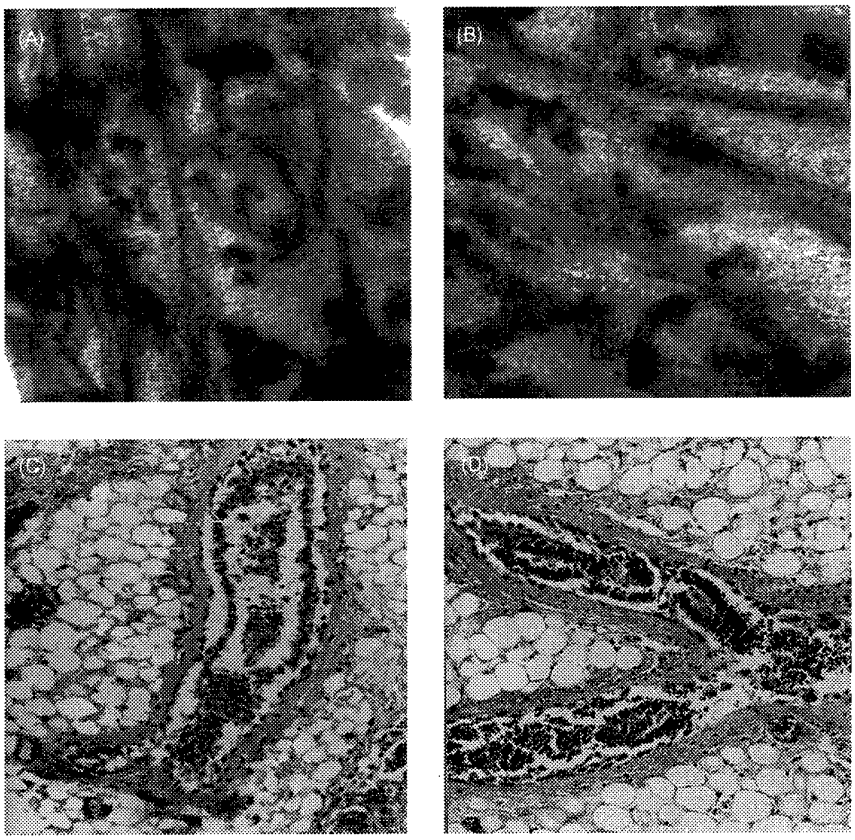

Fig. 7. The 6-day mouse mammary organ culture (MMOC) was performed without the carcinogen treatment. The data shows similar growth in both the control and $1\alpha(OH)D_5$ treated glands. (A) control; (B) $1\alpha(OH)D_5$; fixed and stained with carmine; (C) control and (D) $1\alpha(OH)D_5$, fixed, sectioned, and stained with H and E.

condensed chromatin and reduced Ki 67 expression after 72-h incubation with $1\alpha(OH)D_5$ (Mehta, unpublished data). Taken together, these results indicate that, in human breast epithelial tissues, $1\alpha(OH)D_5$ is selective for its effects on pre-cancerous or cancer cells but shows no effect on normal breast epithelial cell growth.

3.3. Mechanism of $1\alpha(OH)D_5$ action

The effects of $1\alpha(OH)D_5$ have also been evaluated in several breast cancer cell lines [37]. Although these studies do not focus directly on chemoprevention, they do provide excellent insight into the mechanism of action of $1\alpha(OH)D_5$ and its efficacy as an anti-proliferative agent. We had reported that, in ER^+, PgR^+, breast cancer cells, $1\alpha(OH)D_5$ inhibited cell growth by inducing apoptosis as well as differentiation, whereas in ER^- but VDR^+ cells, it induced cell differentiation without the induction of apoptosis [35]. Similar results have also been reported by numerous investigators using other analogs of Vitamin D [38]. The data from these studies consistently reported that breast cancer cells expressing VDR respond to Vitamin D analogs. These results suggested that the mode of action of $1\alpha(OH)D_5$ depended not only on expression of VDR but also on the expression of ER and ER-inducible genes such as PgR.

The effects of $1\alpha(OH)D_5$ on cell cycle were determined using breast cancer cells. The BT474 cells

Table 3
Effects of $1\alpha(OH)D_5$ on cell cycle phases in breast epithelial cell lines

Types	G1 (%)	S (%)	G2 (%)	G1/G2 (%)
BT474				
Control	60.7	30.5	8.8	6.9
$1,25(OH)_2D_3$	71.6*	22.1	6.3	11.4
$1\alpha(OH)D_5$	85.7*	10.3	4.0	21.4
MCF7				
Control	61.2	28.6	10.1	6.1
$1,25(OH)_2D_3$	71.9*	19.3	8.8	8.2
$1\alpha(OH)D_5$	70.0*	20.4	9.6	7.3
MDAMB435				
Control	22.8	31.3	45.9	0.5
$1,25(OH)_2D_3$	21.1	33.0	45.3	0.5
$1\alpha(OH)D_5$	21.1	23.6	55.3	0.4
MCF12F				
Control	72.4	16.2	11.4	6.4
$1,25(OH)_2D_3$	61.1*	20.2	19.0	3.2
$1\alpha(OH)D_5$	67.3*	16.2	16.5	4.1

* Significantly different from control ($P < 0.05$).

were treated with 1 μM $1\alpha(OH)D_5$ for various time points and processed for FACS analysis. Results showed that 70% of the control cells were distributed in the G1 phase, whereas treatment with $1\alpha(OH)D_5$ induced growth arrest with 84% cells in the G1 phase of the cycle. The results are summarized in Table 3. In agreement with our cell proliferation data, there was no difference between the distribution of cells in various cell cycle stages for MCF12F and MBA-MD-231 cells with $1\alpha(OH)D_5$ treatment. Both MDA-MB-231 and MDA-MB-435 cells are devoid of steroid receptors; therefore, these cells were not expected to respond to $1\alpha(OH)D_5$ treatment. These results further confirm that the action of $1\alpha(OH)D_5$ may be mediated, in part, by VDR.

The mechanism of action of $1\alpha(OH)D_5$ was further evaluated by determining the ability of the cells to undergo apoptosis. The BT474 cells were treated with $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 72 h and then stained with acridine orange and observed under fluorescent microscope for detection of chromatin condensation. Fig. 8 shows that BT474 cells underwent apoptosis with $1\alpha(OH)D_5$ treatment as determined by acridine orange and ethidium bromide staining. The stain distinguishes live cells from those that are undergoing apoptosis. On the other hand, no apoptosis was observed in ER^-, PgR^-, VDR^+, BCA-4 cells, though there was an induction of differentiation as shown by casein, lipids, and α2 integrin expression [35].

Chemopreventive agents are being developed mostly for people who do not yet have disease but are at high risk of developing cancer. Here, we show that the Vitamin D analog might be selective for transformed cells. The population at high risk of developing cancer is assumed to be initiated for carcinogenesis and, as we have shown, initiated cells respond well to $1\alpha(OH)D_5$. In addition, we also showed here that $1\alpha(OH)D_5$ is effective against steroid-responsive cancer cells. These results suggest that $1\alpha(OH)D_5$ can be considered as a possible chemopreventive and therapeutic agent. Moreover, if given in combination with other agents, it may provide synergistic protection.

It is unclear as to where chemoprevention ends and chemotherapy begins. However, the clear principle

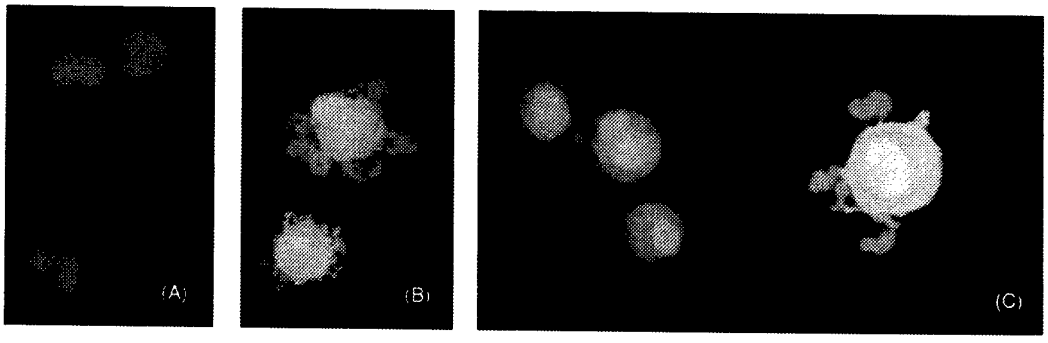

Fig. 8. Induction of apoptosis in BT474 cells by 1 μM $1\alpha(OH)D_5$, as determined by acridine orange and ethidium bromide staining. (A) control; (B) $1,25(OH)_2D_3$ (0.1 μM); (C) $1\alpha(OH)D_5$ (1 μM).

and prerequisite of chemoprevention is that the agent should not have any adverse effects. The lack of toxicity of $1\alpha(OH)D_5$ at an effective concentration may provide a rationale for its role in chemoprevention and therapy.

In summary, we have described here the chemopreventive properties of a relatively new non-toxic analog of Vitamin D, $1\alpha(OH)D_5$, against mammary carcinogenesis models. In addition, our results suggest that $1\alpha(OH)D_5$ may be active selectively against transformed cells without showing adverse effects on normal breast epithelial cells.

Acknowledgements

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Efficacy and Mechanism of Action of 1α-hydroxy-24-ethyl-Cholecalciferol (1α[OH]D5) in Breast Cancer Prevention and Therapy

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Abstract

It is now well established that the active metabolite of vitamin D_3, 1α,25$(OH)_2D_3$, regulates cell growth and differentiation in various in vitro cancer models. However, its clinical use is precluded due to its hypercalcemic activity in vivo. Hence, several less calcemic vitamin D analogs have been synthesized and evaluated for their chemopreventive and therapeutic efficacy in experimental carcinogenesis models. A novel analog of vitamin D_3, 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5), has currently been under investigation in our laboratory for its application in breast cancer prevention and therapy. 1α(OH)D5 had been shown to inhibit development of estrogen- and progesterone-dependent ductal lesions as well as steroid hormone-independent alveolar lesions in a mammary gland organ culture (MMOC) model. Moreover, the inhibitory effect was more significant if 1α(OH)D5 was present during the promotional phase of the lesion development. The growth inhibitory effect of 1α(OH)D5 has also been manifested in several breast cancer cell lines, including BT-474 and MCF-7. Breast cancer cell lines that responded to 1α(OH)D5 treatment were vitamin D receptor positive (VDR+). Vitamin D receptor-negative (VDR−) cell lines, such as MDA-MB-231 and MDA-MB-435, did not show growth inhibition upon incubation with 1α(OH)D5. This suggests the requirement of VDR in 1α(OH)D5-mediated growth effects. Interestingly, breast cancer cells that were VDR+ as well as estrogen receptor positive (ER+) showed cell cycle arrest and apoptosis, while VDR+ but ER− cells (UISO-BCA-4 breast cancer cells) showed enhanced expression of various differentiation markers with 1α(OH)D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly down-regulated in ER+ BT-474 cells with 1α(OH)D5 treatment. This implies a differential effect of 1α(OH)D5 on ER+ vs. ER− cells. Additionally, comparison between the effects of 1α(OH)D5 on normal vs. transformed cells indicated that 1α(OH)D5 does not suppress cell prolifera-

Recent Results in Cancer Research, Vol. 164

tion of normal epithelial cells but selectively targets growth of transformed cells. We extended our experiments to determine in vivo effects of 1α(OH)D5 using an MNU-induced mammary carcinogenesis model in female Sprague-Dawley rats. Results showed that 1α(OH)D5 (25–50 μg/kg diet) decreased the incidence and multiplicity of mammary tumors in these rats. In addition, it increased the latency period of early precancerous lesions. Similar studies, with DMBA as a carcinogen in younger rats, showed that 1α(OH)D5 supplementation was effective in reducing onset of carcinogenesis in rats and the effect was largely reflected during the promotional phase of carcinogenesis. Recently, a preclinical toxicity profile for 1α(OH)D5 was completed in rats and dogs that provides estimation of the maximum tolerated dose in mammals. Based on our findings, we will shortly be initiating a 1α(OH)D5 phase I clinical trial for breast cancer patients.

Introduction

Breast cancer is generally characterized by transformation of normal to atypical hyperplastic epithelium, with subsequent risk of progression to intraductal carcinoma and in some cases invasion into stroma (Mallon et al. 2000). Breast cancer is the second leading cause of cancer-related deaths among women in the US, with about 180,000 new cases and 46,000 deaths annually (Edwards et al. 2002). Although the overall incidence of breast cancer has not been reduced in the last decade, the breast cancer-related mortality has been decreasing with approximately 3.4% annual decrease from 1995 through 1998 in the US (Howe et al. 2001; Peto et al. 2000). This decrease in mortality is probably a result of availability of greater screening efficiency and better chemopreventive and therapeutic strategies. Despite increased survival rates, breast cancer results in considerable morbidity and patient care costs. Chemoprevention is an important aspect of curbing the progression or recurrence of the disease. The chemopreventive agents usually include natural or synthetic compounds that can either prevent transformation or inhibit proliferation of transformed cells by inducing apoptosis, growth arrest or differentiation of initiated and transformed cells (Rosenbaum-Smith and Osborne 2000). Several classes of compounds have been under investigation in this regard. These include selective estrogen receptor modulators, retinoids, deltanoids (vitamin D derivatives), phytoestrogens, flavonoids, and aromatase inhibitors, among others (Kelloff et al. 1996).

On a global basis, breast cancer incidence is fivefold higher among middle-aged women in the Western countries than in women from Asian countries. Various diet and lifestyle as well as genetic factors have been implicated in the high occurrence of breast cancer in the Western world. Some epidemiological studies have shown association of lower sunlight exposure to higher breast, colon, and prostate cancer mortality rates in the US and other Western countries (Freedman et al. 2002; Polek and Weigel 2002; Garland et al. 1990; Gorham et al. 1990). This is consistent with reports of an association of breast

cancer mortality with lower serum vitamin D_3 levels (John et al. 1999; Christakos 1994). Lower serum vitamin D_3 levels could be due to lower sunlight exposure as well as lower dietary intake.

The biologically active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$ or calcitriol, is a steroid hormone that was identified in the early 1920s as an antirachitic substance (Carpenter and Zhao 1999). Later it was established that vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol by the action of ultraviolet radiation. Vitamin D_3 is activated subsequently in liver and kidney by the hydroxylation reactions at C25 and 1α positions to yield $1\alpha,25(OH)_2D_3$. Calcitriol has been known to exert calciotrophic effects, mainly through increasing calcium uptake in the intestine for regulation of bone health. Aside from its role in calcium homeostasis, vitamin D_3 is involved in regulation of various cellular processes. Vitamin D_3 binds to nuclear vitamin D receptor (VDR) and undergoes conformational changes, which allow VDR to function as a transcription factor (Jones et al. 1998; Haussler 1986). Earlier, VDR was found to be present in abundance in intestine, bone, liver, and kidney cells. Aside from the classic target organs, VDR has now been isolated from a variety of tissues, including normal mammary epithelium as well as breast tumors (Friedrich et al. 1998; Buras et al. 1994; Eisman et al. 1980).

In order for VDR to function, it needs to interact with vitamin D response elements (VDRE) and bind to DNA to initiate or repress transcription (Pike 1991). VDR must form a dimer to stabilize VDRE transactivation (Jones et al. 1998). Most common partners for VDR heterodimerization are nuclear accessory factor (NAF) and retinoid X receptor (RXR) (Rachez and Freedman 2000). VDR transactivation of VDRE results in regulation of a wide variety of genes, some of which are involved in cell growth and proliferation. Vitamin D_3 also exerts some nongenomic rapid responses possibly through a putative membrane receptor (Falkenstein et al. 2000).

The presence of VDR in the normal mammary epithelial cells suggests a role of calcitriol in the regulation of mammary gland function. The levels of VDR in mammary tissue increase during pregnancy and lactation and decrease as the glands regress back to normal size (Zinser et al. 2002; Narvaez et al. 2001). VDR knockout mice have been shown to have larger mammary glands than normal mice; it has also been shown that the glands would not regress back to prepregnancy size at the termination of lactation (Zinser et al. 2002). This suggests that vitamin D mediated signaling may be very important for maintaining the normal cycling of the mammary gland. Various case studies indicate that a high percentage (60%–80%) of breast cancer epithelia contain VDR (Friedrich et al. 1998) and that there is a positive correlation between VDR polymorphisms and increased risk of breast cancer (Bretherton-Watt et al. 2001; Lundin et al. 1999). These reports further signify vitamin D_3-mediated signaling to be of importance in regulating healthy mammary gland. In cell culture models, vitamin D_3 has been demonstrated as an inducer of growth arrest and differentiation in various cancer cell lines, including breast cancer cells (Hisatake et al. 2001; Welsh et al. 1998; James et al. 1997). Taken together, these results warrant potential use of vitamin D_3 in cancer preven-

tion and therapy. However, due to its hypercalcemic activity, vitamin D_3 can not be administered at doses that would be effective for chemoprevention or therapy. Adverse effects of vitamin D_3 at cancer-preventive doses are hypercalcemia, soft tissue calcification, weight loss, and possibly death (Roder and Stair 1999; Vieth, 1999).

Since the early 1980s, there has been a search for a vitamin D_3 analog that would selectively modulate VDR to produce growth-regulating effects without interfering with the calcium metabolism. Several analogs have been synthesized and tested for this purpose; but only a few have shown promising results in cell culture and animal models. Vitamin D_3 analogs currently being evaluated for breast cancer prevention include seocalcitol (EB-1089), calcipotriol (KH-1060), Maxacalcitol (OCT), RO-24-5531, and 1α(OH)D5 (Mehta and Mehta 2002; Guyton et al. 2001). In this review, we summarize the results from experiments conducted in our laboratory that elucidate the potential role of 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5) in breast cancer prevention or therapy.

Synthesis and Characterization of Vitamin D Analog, 1α(OH)D5

As mentioned earlier, vitamin D_3 can be obtained from food as well as synthesized in the skin through the action of sunlight. Vitamin D_3 belongs to the family of 9,10-secosteroids which differ only in side-chain structure (Napoli et al. 1979). Other forms of D-compounds include D2, D4, D5, and D6. In the late 1970s, major interest in the synthesis of these compounds was to evaluate them for use in management of renal osteodystrophy and osteoporosis. In this regard the calcemic activity of D series of compounds was compared and D5 was found to be the least calcemic of all (Napoli et al. 1979), a property that would later prove useful in its possible application for cancer prevention. The D5 form is also known as irridated 7-dehydrositosterol. The hydroxylated form of D5 (1α[OH]D5) was synthesized as described previously (Mehta et al. 1997a).

Briefly, β-sitosterol acetate was converted to 7-dehydro-β-sitosterol acetate by allelic bromination and dehydrobromination. Lithium aluminum hydride and tetrahydrofurane were used to reduce 7-dehydro-β-sitosterol to 7-dehydro-3β-sitosterol. The reaction mix was sequentially subjected to photolysis and thermolysis to yield 24-ethyl-cholecalciferol (D5). D5 was hydroxylated by Paaren-DeLuca hydroxylation sequence to produce 1α(OH)D5. The product was crystallized and characterized by ^{1}H nuclear magnetic resonance at 400 Hz and mass spectroscopy. The purity was assessed by high-pressure liquid chromatography. The following properties were observed: melting point, 150–152°C; UV λ-max, 265 nm; molar extinction coefficient (ε), 18000; molecular weight, 428.7. The major structural differences between biologically active vitamin D_3 and 1α(OH)D5 are the lack of hydroxylation at the C-25 position and the presence of an ethyl group at the C-24 position in the 1α(OH)D5 molecule (Fig. 1).

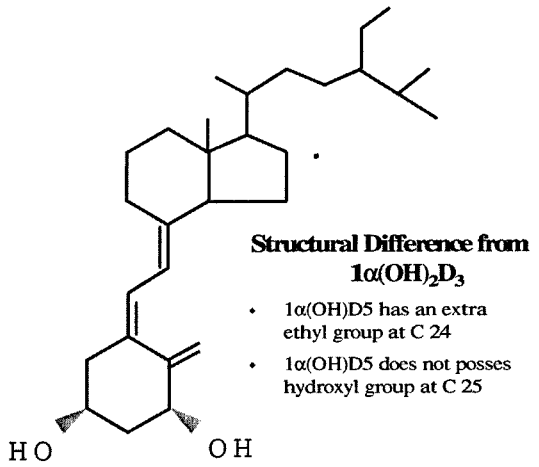

Fig. 1 Structure of 1α(OH)D5 and its Ca^{++} mobilizing activity in mammals in relation to other primary vitamin D series compounds

Calcemic Activity of 1α(OH)D5

Earlier studies in DeLuca's lab had shown that among the known vitamin D series of compounds (vitamin D_2–D_6), D5 is the least calcemic of all (Napoli et al. 1979). D5 was found to be 80-fold less active than vitamin D_3 in the intestine and about 100- to 200-fold less active in bone in mobilizing the Ca^{++} stores (Napoli et al. 1979). The calcemic activity of the hydroxylated form was not known. Therefore, we measured calcemic activity as well as body weight change in animal models to determine the maximum tolerable dose and toxicity of 1α(OH)D5. In the first experiment, 3-week-old Sprague-Dawley male rats were fed a vitamin D_3-free diet containing 0.47 g calcium and 0.3 g phosphorus/100 g diet (Mehta et al. 1997a). These rats were kept under yellow light to create a vitamin D_3-deficiency state. After the rats were fed a vitamin D_3-deficient diet for 3 weeks, their plasma calcium levels were measured and rats with calcium levels under 6.0 mg/dl were considered vitamin D_3 deficient. Vitamin D_3-deficient rats were administered 1α(OH)D5 intragastrically for 14 days and the plasma calcium levels were measured. The control group showed a plasma calcium concentration of 5.4+0.3 mg/dl, while the rats receiving 1α(OH)D5 at a dose of 0.042 μg/kg per day had plasma calcium concentration of 6.0+0.63 mg/dl, which was not significantly different from the control rats (Mehta et al. 1997a). On the other hand, vitamin D_3 increased

Table 1 Calcemic activity of 1α(OH)D5 in Sprague-Dawley rats

Treatment	Sample size	Dose	Plasma Ca^{++} (mg/dl)
Vitamin D-deficient male rats		(μg/kg body weight)	
Control	8	0.0	5.4±0.28
1α(OH)D5	8	0.042	6.0±0.63
1α(OH)$_2$D$_3$	8	0.042	8.1±1.2*
Vitamin D-sufficient female rats		(μg/kg diet)	
Control	15	0.0	7.0±1.19
1α(OH)D5	15	25.0	7.4±1.10
1α,25(OH)$_2$D$_3$	15	12.8	8.5±1.17*

* Significantly different from control (p <0.05)

plasma calcium concentration 50% over that of the control group (Table 1). During these experiments, the 1α(OH)D5 group did not differ in total body weight from control group. No other signs of toxicity were observed in 1α(OH)D5-fed rats compared to controls.

In a separate experiment, female Sprague-Dawley rats were fed a diet supplemented with 1α(OH)D5 to determine its calcemic activity in vitamin D_3-sufficient rats. Food was provided ad libitum. There was no body weight change at 50 μg 1α(OH)D5/kg diet in vitamin D_3-sufficient rats, while a dose of 12.8 μg 1α,25(OH)$_2$D3/kg diet was sufficient to bring about significant weight loss in the animals (Table 1). Maximum tolerated dose was determined to be 50 μg/kg diet, based on the weight and calcemic activity of 1α(OH)D5 in these rats (Mehta et al. 2000a). In addition to these experiments, we also conducted toxicity studies under the GLP using rats and dogs. For rats, the dose at which signs of toxicity first appeared was 10 μg/kg body weight (equivalent to 100 μg 1α(OH)D5/kg diet for a 150-g rat), which is twice the amount needed to bring about effective chemoprevention. However, the dogs had much lower tolerance for 1α(OH)D5 compared to rats. Based on these results, we are now conducting further studies to determine the appropriate and safe dose of 1α(OH)D5 for use in clinical settings.

Since vitamin D_3 exerts most of its effects through binding to VDR, we evaluated the ability of 1α(OH)D5 to bind to VDR. The binding affinity of 1α(OH)D5 to VDR was determined using competitive binding assays (unpublished data). Results showed that the binding affinity of 1α(OH)D5, in competition with radioactive 1α,25(OH)$_2$D$_3$ to purified VDR ligand-binding domain is 1000-fold less than 1α,25(OH)$_2$D$_3$ (Fig. 2). The IC_{50} for 1α(OH)D5 was 100 pM, while for 1α,25(OH)$_2$D$_3$, it was 0.08 pM. The lower binding affinity may explain the decreased calcemic activity of 1α(OH)D5. However, due to its lower calcemic activity, 1α(OH)D5 can be administered at much higher doses than 1α,25(OH)$_2$D$_3$. This quality can allow use of 1α(OH)D5 for prevention in the general population as well as high-risk groups. It is also important to note that the in vivo VDR affinity to its ligand is tissue specific (Napoli et al. 1979), which could not be manifested in our experiments that were conducted using

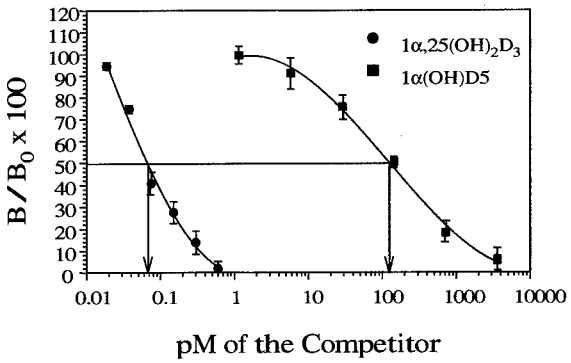

Fig. 2 Binding affinity of 1α(OH)D5 to VDR in comparison with 1α,25(OH)$_2$D$_3$

purified VDR. We have not yet critically evaluated metabolism and pharmacokinetics of 1α(OH)D5 in target organs.

Anticarcinogenic Effects of 1α(OH)D5 in In Vitro Models

The effectiveness of a variety of chemopreventive agents has been evaluated by organ culture of the mouse mammary gland (MMOC). The mammary glands from balb/c mice are harvested and cultured in presence of appropriate hormones (Mehta et al. 1997b). These glands are subjected to short stimulation with a carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA), which results in formation of precancerous preneoplastic lesions. When implanted in syngeneic hosts, the epithelial cells from these lesions give rise to adenocarcinomas. Effective chemopreventive agents would inhibit the development of these preneoplastic lesions. The chemopreventive activity of a compound in MMOC correlates very well with the activity in in vivo carcinogenesis models (Mehta et al. 1997b). Using a DMBA-induced MMOC model, Mehta et al. (1997a) showed that 1α(OH)D5 possesses chemopreventive activity. Fifteen mammary glands (per group) from balb/c mice were incubated with appropriate hormones and were exposed to the carcinogen DMBA (2 μg/ml of culture media) on day 3 of a 24-day culture. The group of glands incubated with 1α(OH)D5 showed significant reduction of lesion formation compared to the control group (Fig. 3). Percent inhibition of lesion formation in each treatment group was calculated by comparing the incidences of lesions between the control and the treated group. A dose–response curve showed that 100% inhibition was achieved at 10 μM 1α(OH)D5 concentration, but the optimal dose seems to be 1 μM, as it shows significant (75%) inhibition without any signs of cytotoxicity. Vitamin D$_3$, on the other hand, caused dilation of ducts and disintegration of alveolar structures as signs of toxicity at 1 μM concentration. Based on the MMOC model, 1 μM 1α(OH)D5 seems to be equivalent in potency to 0.1 μM 1α,25(OH)$_2$D$_3$.

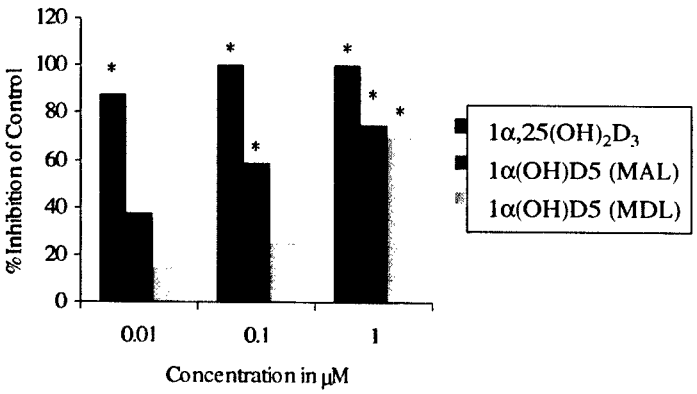

Fig. 3 Chemopreventive efficacy of 1α(OH)D5 in inhibiting mammary alveolar (MAL) and ductal (MDL) lesions in mouse mammary gland organ culture in comparison to 1α,25(OH)$_2$D$_3$

In order to establish the stage specificity for the effectiveness of 1α(OH)D5 in a DMBA-induced MMOC model, 1α(OH)D5 was added either prior to or subsequent to carcinogen treatment. The initiation-only group received 1α(OH)D5 for the first 4 days of culture, whereas the promotion-only group received the treatment after withdrawal of carcinogen (days 4–10). Results indicated that 1α(OH)D5 is more effective when present during the promotional stages of lesion formation (Mehta et al. 2000a). In addition to inhibition of lesion formation, 1α(OH)D5 was effective in inducing VDR and TGFβ1 expression in mammary epithelial cells of MMOC. VDR and TGF-β1 expression was measured using immunohistochemistry. Briefly, paraffin-embedded sections were rehydrated, fixed, permeabilized, and incubated with primary antibody. The primary antibody binding was detected using biotinylated link and peroxidase-conjugated streptavidin, which was then visualized by 3-amino-9-ethylcarbazole as chromogen. The mammary epithelial cells, which stained negative for VDR, failed to show TGF-β1 induction upon 1α(OH)D5 treatment. This implies the involvement of VDR in 1α(OH)D5-mediated effects. The extent of induction of VDR and TGF-β1 upon treatment with 1.0 μM 1α(OH)D5 was similar to that observed with 0.1 μM vitamin D$_3$ (Mehta et al. 1997a). Despite the 1000-fold lower affinity of 1α(OH)D5 for VDR in comparison to 1α,25(OH)$_2$D3, its chemopreventive activity is equivalent to 1α,25(OH)$_2$D$_3$ at only a 100-fold higher concentration. Therefore, it seems likely that the antiproliferative effects of 1α(OH)D5 may not be dependent solely upon its in vitro interactions with VDR.

Since the MMOC experiments involved the whole organ, the actions of 1α(OH)D5 on breast epithelia itself were not clearly established. Hence, we tested the growth effects of 1α(OH)D5 on various breast cancer cell lines of epithelial origin. All the cell lines tested were purchased from ATCC (Manassas, VA, USA), except UISO-BCA-4 cells. This cell line was established in our laboratory from metastatic pleural fluid obtained from a 56-year-old woman with a confirmed diagnosis of breast carcinoma (Mehta et al. 1992). The

Table 2 Growth response of various breast cancer cell lines to 1α(OH)D5 treatment

Cell lines	VDR status	ER status	PR status	Inhibition (%) [a]	Net effect of 1α(OH)D5
BT-474	+	+	+	50	Cell cycle arrest, apoptosis
MCF-7	+	+	+	45	Cell cycle arrest, apoptosis
ZR-75–1	+	+	+	30	Growth inhibition
T-47D	+	+	+	30	Growth inhibition
UISO-BCA-4	+	–	–	40	Growth inhibition, differentiation
MDA-MB-231	–/+ (?)	–	–	None	None
MDA-MB-435	–	–	–	None	None

[a] Percent growth inhibition at 1 μM 1α(OH)D5 for 72 h, adjusted for control

growth effects of 1α(OH)D5 were assessed on BT-474, MCF-7, ZR-75–1, T-47D, UISO-BCA-4, MDA-MB-231 and MDA-MB-435 cell lines using multiple measures: cell counter, MTT absorbance assay (Twentyman and Luscombe 1987), and cell cycle analysis with propidium iodide staining and flow cytometry (Vindelov et al. 1983). The overall effects of 1α(OH)D5 on the growth of different cell lines are summarized in Table 2. All the cell lines that were positive for VDR showed significant growth inhibition ($p<0.05$) after 72 h of incubation with 1α(OH)D5. BT-474, and MCF-7 (VDR+ ER+ PR+) cells showed the greatest growth inhibition and G-1 cell cycle arrest upon 1α(OH)D5 treatment. Similarly, UISO-BCA-4 (VDR+ ER– PR–) cells exhibited growth inhibition in response to 1α(OH)D5 treatment. On the other hand, VDR– MDA-MB-231 and MDA-MB-435 cells did not show any growth inhibition at 1 μM 1α(OH)D5 treatment (Mehta et al. 2002). The dose–response curve for 1α(OH)D5 effect in BT-474 cells was similar to that observed in the MMOC experiments.

Chemopreventive Efficacy of 1α(OH)D5 in In Vivo Carcinogenesis Models

Once we established the in vitro efficacy of 1α(OH)D5, the effects of 1α(OH)D5 were evaluated in experimental mammary carcinogenesis models. We used mammary-specific carcinogen *N*-methyl-*N*-nitrosourea (MNU) in rats to induce tumors and evaluated the efficacy of 1α(OH)D5 to prevent or delay the incidence of mammary cancers in these rats (Mehta et al. 2000a). Fifteen Sprague-Dawley female virgin rats per group (9 weeks old) were fed 1α(OH)D5-supplemented diet (25 or 50 μg/kg) for 2 weeks before the carcinogen treatment. The carcinogen MNU was given as a single intravenous injection of 50 mg acidified MNU/kg body weight at 80 days of age. The rats continued to receive the 1α(OH)D5-supplemented diet until they were killed at 190 days of age. The tumor incidence in control rats was 80%, which, compared to controls, decreased in 25- and 50-μg/kg diet group by 33% and 42%, respectively (Table 3). The tumor incidence in the low-dose group was not sig-

Table 3 Efficacy of 1α(OH)D5 in preventing carcinogenesis in animal models

Tissue	Sample	Dose	Duration[a]	Tumor incidence	Multiplicity
MNU-induced tumors	15	0.0	17	80%	1.6
in rats	15	50 μg/kg diet	17	47%[a]	0.8[a]
DMBA-induced tumors	20	0.0	22	85%	1.9
in rats	20	20 μg/kg diet	22	40%[a]	1.3
UISO-BCA-4 xenograft	5	0.0	6	100%	NA
in athymic mice	5	8 ng (s.c.)[b]	6	0%[c]	NA
UISO-BCA-4 xenograft	5	0.0	6	100%	NA
in athymic mice	5	12.5 μg/kg diet	6	0%[a]	NA
BT-474 xenograft	5	0.0	8.5	0.01 cm^3	NA
in athymic mice	5	12.5 μg/kg diet	8.5	0.125 cm^3[c]	NA

[a] Duration in weeks

[b] 8 ng 1α(OH)D5 subcutaneously injected thrice weekly for 60 days

[c] Significantly different from control ($p<0.05$)

[d] Results are expressed as tumor volume (cm^3)

nificantly reduced from control ($p=0.12$), whereas the high-dose group had a significantly lower tumor incidence ($p=0.03$). However, when the three groups were compared using log-rank analysis, the comparison reached statistical significance ($p=0.0495$). Tumor multiplicity was not significantly different between the control group and the 25-μg/kg diet group, but it was significantly lower in the high-dose group ($p=0.02$).

The encouraging results from MNU-carcinogenesis model prompted us to extend our in vivo experiments. Since MNU is a direct-acting carcinogen, we chose another mammary-specific carcinogen that needs to be metabolized, such as DMBA. For the DMBA carcinogenesis study, 7-week-old rats (20 per group) were given 15 mg DMBA intragastrically. 1α(OH)D5 was supplied in the diet (20–40 μg/kg diet) 2 weeks prior to carcinogen treatment. The control group showed 85% tumor incidence and the high-dose group showed 60% incidence, while the low-dose group showed a significant decrease in incidence (40%). Table 3 summarizes the results from in vivo experiments. Although the high-dose group did not show a significant decrease in tumor incidence, it had significantly lower tumor multiplicity (0.6 compared to 1.9 in the control group). Moreover, the chemopreventive efficacy of 1α(OH)D5 was more pronounced when provided at progressional stages of the disease.

In addition to assessing chemopreventive properties of 1α(OH)D5 in mammary carcinogenesis, we evaluated its efficacy as a possible chemotherapeutic agent. These experiments were carried out in xenograft models, as previously described (Mehta and Mehta 2002). Initial studies were conducted using xenograft of UISO-BCA-4 cells pretreated with 1 μM 1α(OH)D5 for 10 days, which failed to form tumors in athymic (4-week-old) mice. In other studies, UISO-BCA-4 cells were xenografted in athymic mice and either 8 ng 1α(OH)D5 per animal was injected IP thrice a week or 1α(OH)D5 was provided in the diet at

12.5 μg/kg diet for 6 weeks. All the animals in the control group formed tumors whereas only one of the treated animals showed a scab-like structure at injection site in the IP group. Forty percent of controls showed metastasis to lymph nodes but 1α(OH)D5 treatment prevented metastasis of cells transplanted in athymic mice (Mehta and Mehta 2002). In the dietary treatment group, 1α(OH)D5 inhibited growth of UISO-BCA-4 cells and the tumor volume was suppressed to nearly 50% of control. Similar results were obtained with BT-474 xenograft in athymic mice. These results suggest that 1α(OH)D5-induced cell growth inhibition and differentiation is protective against tumor growth in the xenograft model as well.

Growth Response of Normal versus Transformed Cells to 1α(OH)D5

While we established that 1α(OH)D5 has growth inhibitory action on cancer cells, the effects on normal breast epithelial cells were not known. In order to determine that, we cultured mammary glands from mouse with appropriate hormones in the absence of any carcinogens. Ten glands were treated with 1α(OH)D5 and other glands were used as controls. At the end of 6-day culture, the glands were terminated, paraffin embedded, and sectioned for pathological evaluation. Histopathological examination showed no difference in the growth and morphology of glands treated with 1α(OH)D5 from that of control glands. In view this result, we evaluated the effects of 1α(OH)D5 on MCF-12F cells, which are nontumorigenic breast epithelial cells derived from reduction mammoplasty from a 60-year-old Caucasian woman. These cells were spontaneously immortalized by long-term culture in low-Ca^{++} media. To determine their growth response, MCF-12F cells were incubated with 1α(OH)D5 for various intervals, but no growth inhibitory effect was observed at the 1-μM concentration.

To establish selectivity of 1α(OH)D5 effects on transformed or preneoplastic cells, we transformed MCF-12F cells with DMBA and MNU to study if the transformation status could affect the response to 1α(OH)D5. Transformation was performed using the protocol described elsewhere (Lazzaro et al. 1997). Briefly, passage 10 MCF-12F cells were grown to subconfluency in tissue culture dishes and incubated with DMBA (2 μg DMBA/ml culture media) for 24 h. The procedure was repeated the next day. Extensive cell death resulted. The surviving cells were allowed to grow in fresh medium and later selected out with serum starvation. The resulting cell line was designated MCF-$12F_{DMBA}$. Similarly, in another experiment, MNU was dissolved in acidified saline (pH 5.3) and added to subconfluent MCF-12F cells at a concentration of 25 μg/ml twice daily for 2 days. The surviving cells were allowed to grow and the new cell line was established after serum starvation. These cells were called MCF-$12F_{MNU}$. The growth rate and morphological characteristics were compared between these cell lines. The growth rates of transformed cells were three times higher than MCF-12F. By the fifth passage of postcarcinogen treatment, the MCF-$12F_{DMBA}$ doubling time was reduced to one-third of MCF-12F,

Table 4 Growth effects of 1 μM 1α(OH)D5 on normal and transformed MCF-12F breast epithelial cells

Cell line	Treatment	Cell count	Cell cycle (% G-1)	MTT absorbance
MCF-12F	Control	47,250±474	68	0.045±0.06
	1α(OH)D5	45,820±587	71	0.044±0.04
MCF-12F$_{MNU}$	Control	91,800±120	43	0.185±0.06
	1α(OH)D5	73,616±138*	65	0.078±0.01*
MCF-12F$_{DMBA}$	Control	105,470±42.4	49	0.128±0.02
	1α(OH)D5	8,035±91*	67	0.075±0.01*

* Significantly different from control ($p<0.05$)

while for MCF-12F$_{MNU}$, it was reduced to one-fourth of MCF-12F. Moreover, the transformed cell lines did not exhibit the contact inhibition characteristic of the normal cells.

As mentioned earlier, MCF-12F cells showed no growth inhibitory response with 1α(OH)D5 treatment. The transformed cells, on the other hand, showed significant growth inhibition (60% for MCF-12F$_{MNU}$ and 40% for MCF-12F$_{DMBA}$), as determined by the MTT absorbance assay. Other measures of growth provided similar results (Table 4). These studies indicate that the transformed cells respond differently to 1α(OH)D5 treatment than the parent cell line.

Potential Mechanism of Action of 1α(OH)D5 in Breast Cancer Prevention and Therapy

Previously mentioned studies have implicated the involvement of VDR in 1α(OH)D5-mediated growth effects. VDR− highly metastatic cells such as MDA-MB-231 and MDA-MB-435 do not respond to 1α(OH)D5 treatment. Moreover, mammary epithelial cells which lack VDRs also fail to respond to 1α(OH)D5 and do not show induction of VDR and TGF-β1 (Mehta et al. 1997a). VDR+ breast cancer cells, such as T-47D, had been shown to increase transcription of VDR upon incubation with 1α(OH)D5 as determined by RT-PCR (Lazzaro et al. 2000). This VDR induction was not observed in the cell line BT-474, either at transcription or expression levels, upon treatment with 1α(OH)D5. A possible explanation could be the high constitutive levels of VDR present in this cell line. To ascertain VDR-mediated VDRE transactivation activity of 1α(OH)D5, we used the CAT reporter gene containing VDRE (VDRE-tk-CAT). For this purpose, CV-1 monkey renal cancer cells were used as these lack a functional VDR. After VDRE-tk-CAT transient transfection into CV-1 cells, 1α(OH)D5 could not induce the CAT activity in these cells. But when the cells were cotransfected with VDRE and VDR, there was an enhanced expression of CAT activity, suggesting the capability of 1α(OH)D5 to activate VDR-mediated signaling. The relative CAT activity in CV-1 cells that had been cotransfected with VDRE and VDR was 200,000-fold higher than control when treated with 0.1 μM 1α(OH)D5 (Lazzaro et al. 2000).

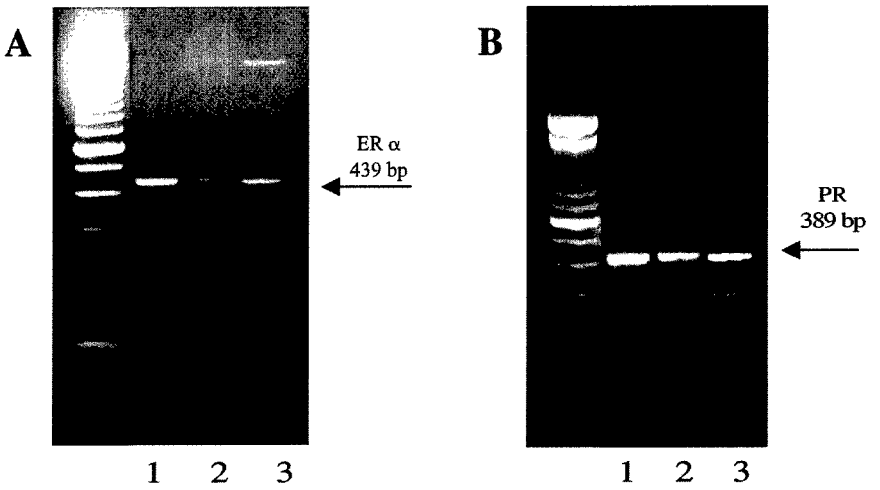

Fig. 4 Down-regulation of estrogen- (**A**) and progesterone- (**B**) receptor transcription with vitamin D_3 and its analog in BT-474 cells as determined by RT-PCR. *Lane 1* control, *lane 2* 1α,25$(OH)_2D_3$, *lane 3* 1α(OH)D5

Breast cancer UISO-BCA-4 cells are ER− and PR−, but VDR+. These cells responded differently to 1α(OH)D5 than the ER+ cells (Mehta et al. 2003). UISO-BCA-4 cells were treated with 0.1 μM 1α(OH)D5 for 10 days. The 1α(OH)D5 treatment resulted in induction of intracytoplasmic casein granules, increased lipid droplets, ICAM-1, α2-integrin, nm23, and VDR, manifesting the differentiation markers. Use of this cell line allows us to determine estrogen-independent effects of 1α(OH)D5. While 1α(OH)D5 induced differentiation in ER− cells, it induced apoptosis in ER+ BT-474 and MCF-7 cells, as determined by acridine orange/ethidium bromide staining and TUNEL assay (Mehta et al. 2003). In both these cell lines, there is a G-1 cell cycle arrest followed by apoptosis.

Because the actions of 1α(OH)D5 differ in ER+ breast cancer cells, we examined the effects of 1α(OH)D5 on estrogen-dependent signaling in the ER+ PR+ BT-474 cells. BT-474 cells showed down-regulation of both ER and estrogen-inducible PR transcription upon treatment with 1α(OH)D5, as determined by RT-PCR (Fig. 4). This was in turn followed by down-regulation at the expression level, as estimated by immunocytochemistry (Fig. 5). These results are consistent with reports by other researchers that describe the role of vitamin D_3 in down-regulation of estrogen-inducible genes (Swami et al. 2000; Stoica et al. 1999). The vitamin D_3-VDR pathway may be a negative feedback mechanism to regulate the estrogen-induced proliferation of the mammary tissue. Some researchers have postulated an interaction of VDR-D_3 to ERE to repress the estrogen-mediated gene transcription (Welsh et al. 1998; Demirpence et al. 1994).

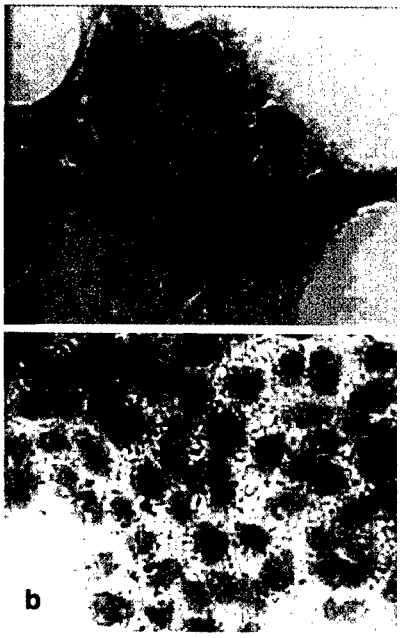

Fig. 5a, b Down-regulation of progesterone receptor (PR) expression with 1α(OH)D5 treatment in BT-474 cells as detected by immunocytochemical analysis. Percentage of cells stained positive for PR were 78% in control (**a**) and 46% in treated cells (**b**)

Since vitamin D_3 is known to regulate a wide variety of genes, we investigated other potential gene targets of 1α(OH)D5 in BT-474 cells. The microarray was performed using Human UniGene 1 by Incyte Genomics, Inc. (Palo Alto, CA, USA), which contained 8,000 genes along with appropriate controls. Among the major targets of 1α(OH)D5 were the estrogen-inducible genes PR, trefoil factor 1 (pS2), and trefoil factor 3 ($p<0.05$). A few selected genes that were statistically significantly altered are presented in Table 5.

As mentioned earlier, the transformed MCF-12F cells showed growth inhibition even though these cells express very low levels of steroid receptors. It is possible that other mechanisms are at work to bring about growth arrest in MCF-12F_{DMBA} and MCF-12F_{MNU} cells. Therefore, we used Clontech Atlas microarrays (Genomics Inc.) with 10,000 genes to identify differentially expressed genes in the transformed MCF-12F_{MNU} cells as compared to the MCF-12F parent cell lines. In a second comparison, we assessed the genes differentially expressed by 1α(OH)D5 treatment in MCF-12F_{MNU} cells. Interestingly, many genes that were differentially expressed in MCF-12F_{MNU} cells compared to the MCF-12F cells were altered inversely in 1α(OH)D5 treated MCF-12F_{MNU} cells (Table 5). Most of the genes that were affected were transcription-related and mitochondrial genes. Of interest are proteins such as vimentin, prohibitin, MAPK-7, and HSP-27, which are usually expressed at higher levels in mammary tumors (Atanaskova et al. 2002; Zajchowski et al. 2001; Storm et al. 1996). These proteins were down-regulated in 1α(OH)D5-treated cells. Differentiation-related proteins such as integrins and cadherins were up-regulated by 1α(OH)D5 in both BT-474 and MCF-12F_{MNU} cell systems.

Table 5 Microarray analysis to determine effects of 1 μM 1α(OH)D5 and MNU-induced transformation on selected genes

Comparison	Genes up-regulated	Genes down-regulated
BT-474 (control)±1α(OH)D5 Incyte Genomics Human UniGene 1 (8 K)	Cytochrome P450 (vitamin D_3 24-hydroxylase) Caspase 3 Cadherin 18 type 2	Trefoil factor 1 (pS2) Progesterone receptor Trefoil factor 3 MMP-9 Thymidine kinase 2 (mitochondrial) Transcobalamine
MCF-12F (control) vs. MCF-$12F_{MNU}$ Clontech Atlas Arrays (10 K)	TGFα Prohibitin Calpain 4 Pituitary tumor transforming 1 HSP-27 Thioredoxin Kearatin 6A and 6B	E2F-4 Integrins Glutathione peroxidase 4 Ornithine decarboxylase antizyme 1 Cystatin B Tissue inhibitor of metalloproteinase 1 TCTP-1
$MCF\text{-}12F_{MNU}$ (control)±1α(OH)D5 Clontech Atlas Arrays (10 K)	Glutathione peroxidase 4 ornithine decarboxylase antizyme 1 Cystatin B Tissue inhibitor of metalloproteinase 1 TCTP-1 Integrin β4 Cadherin 3 Cathepsin D	Prohibitin Vimentin MAPK-7 Thioredoxin HSP-27

Prohibitin might be a potentially important vitamin D_3-regulated protein, which was found to be more highly expressed in the transformed MCF-12F cells than the parent cell line (data not shown). Some studies have shown high prohibitin levels in tumor tissue and cancer cell lines (Jupe et al. 1996; Asamoto and Cohen, 1994). However, the role of this mitochondrial protein is controversial. Wang and co-workers (1999) have shown its involvement in regulation of the cell cycle, whereas others have shown that the levels do not represent the cell cycle-related functions but rather are indicative of mitochondrial stress (Coates et al. 2001). It is possible that the mitochondrial stress may be indicative of the higher proliferative rates of the transformed cells. Another protein of interest was thioredoxin, which was up-regulated in $MCF\text{-}12F_{MNU}$ cells and down-regulated by 1α(OH)D5 treatment. Thioredoxin is a redox protein with growth factor activity that modulates the activity of several proteins important for cell growth. Some researchers have observed increased thioredoxin transcription and expression in primary human tumors (Matsutani et al. 2001; Berggren et al. 1996). Administration of inhibitors of thioredoxin system has been shown to have antitumor activity in vivo (Kirkpatrick et al. 1999). Moreover, Gallegos and co-workers (1996) reported that transfec-

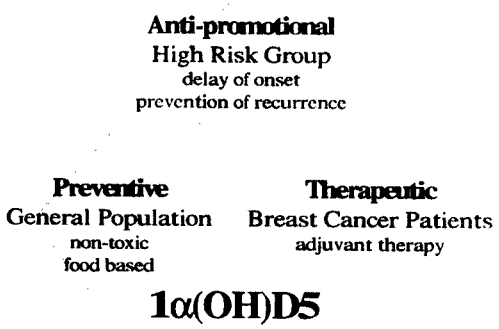

Fig. 6 Potential application of 1α(OH)D5 in breast cancer prevention and therapy

tion of dominant-negative mutant thioredoxin resulted in reversal of transformed phenotype of human breast cancer cells. Therefore, it appears that the mechanism of action of 1α(OH)D5 involves multiple genes and pathways, some of which have not yet been thoroughly investigated. Further studies are needed to elucidate the mechanism of action of 1α(OH)D5 in normal and cancer breast cells.

Conclusions

Results presented in this report on effects of 1α(OH)D5 are suggestive of its promise in chemoprevention. 1α(OH)D5 has consistently been shown to be effective in inhibiting growth of cancer cells as well as preneoplastic lesions in mammary glands in vitro. The in vitro effects are manifested in vivo as well. In the animal carcinogenesis models, 1α(OH)D5 had reduced the incidence of tumors as well as tumor multiplicity, and increased the latency period. Yet there were no changes in total body weight and no apparent signs of toxicity at efficacious doses. More recently, we completed preclinical toxicity studies in rats and dogs under good laboratory practices and regulations, providing an estimation of maximum tolerable dose. The concentration of 1α(OH)D5 required to achieve optimal cell regulatory effects is 100 times higher than the concentration of vitamin D_3. However, there is no hypercalcemia observed at this dose of 1α(OH)D5 to warrant concern. The mechanism of action of 1α(OH)D5 seems to involve VDR as well as cross-talk with the estrogen signaling pathway. It has been shown to inhibit estrogen-induced proliferation. Because of these properties, 1α(OH)D5 might prove suitable in a variety of applications. Furthermore, the differential gene expression profile clearly suggested that the effects of 1α(OH)D5 involve multiple pathways and genes, some of which have not yet been critically studied.

A scheme of possible applications of 1α(OH)D5 is presented in Fig. 6. From a prevention point of view, 1α(OH)D5 might be used in populations that are at high risk or to prevent or delay recurrence of breast tumors in breast cancer

patients. It might also be used in conjunction with other treatments for cancer therapy. Further studies are underway in our laboratory to determine if indeed 1α(OH)D5 would become available for clinical use in the future.

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